THE BIOCHEMICAL JOURNAL

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Edited for

THE BIOCHEMICAL SOCIETY

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Medical Faxon

The Metabolism of Sulphonamides

3. THE DETECTION OF 3-HYDROXYSULPHANILAMIDE IN THE URINE OF HOSPITAL PATIENTS TREATED WITH SULPHANILAMIDE

By R. T. WILLIAMS, *Department of Biochemistry, University of Liverpool*

(*Received* 28 *June* 1946)

3-Hydroxysulphanilamide has been isolated in a pure crystalline condition from the urine of rabbits which had been heavily dosed with sulphanilamide (Williams, 1946). In the present paper it will be shown that 3-hydroxysulphanilamide is also present as a conjugate in the urines of hospital patients treated with sulphanilamide.

The isolation in a pure state of small amounts of 3-hydroxysulphanilamide from urine is a difficult task uncertain of success. Therefore, a colour reaction has been developed for its qualitative detection in urine. This colour reaction appears to be fairly specific under the conditions used here and depends upon the oxidation of 3-hydroxysulphanilamide by dilute ferric chloride to a compound which can be extracted with ether. The ethereal solution has an intense red colour which shows an absorption band ($\lambda_{max.}$, 530 mμ.) in the visible region of the spectrum.

METHODS

The colour reaction for 3-hydroxysulphanilamide

It is well known that phenols react with ferric salts to give highly coloured compounds. Wesp & Brode (1934) have suggested, from spectroscopic and other data, that the colour produced is probably due to complex co-ordinated ions of the type $Fe(OR)_6^{=}$, where OR is ionized phenol.

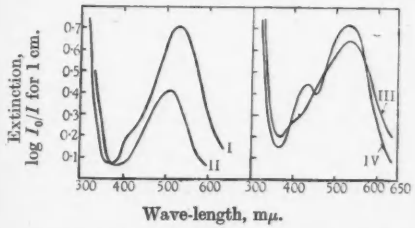
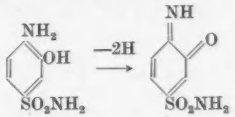


Fig. 1. Absorption spectra of the red ethereal solutions obtained by the action of aqueous ferric chloride in the presence of ether on, I, synthetic 3-hydroxysulphanilamide; II, *p*-aminophenol hydrochloride; III, extracts of human sulphanilamide urine; and IV, extracts of rabbit sulphanilamide urine.

When a few drops of 1 or 2% $FeCl_3$ are added to an aqueous solution of 3-hydroxysulphanilamide, a purplish red colour is obtained which rapidly turns red, then brown, finally giving a brownish cloudy solution. These changes are complete in about a minute after mixing. If, however, the 3-hydroxysulphanilamide solution is first shaken with about 2 vol. of ether and then $FeCl_3$ added and the mixture shaken, the ether layer becomes coloured an intense red (containing some blue) which is comparatively stable; the aqueous layer, however, undergoes the same colour changes as in the absence of ether. The spectral absorption, in the visible region, of this coloured ethereal solution is recorded in Fig. 1, which also gives the result using *p*-aminophenol hydrochloride instead of 3-hydroxysulphanilamide.

Other considerations, however, indicated that the colour produced in ether from 3-hydroxysulphanilamide and $FeCl_3$ was probably not that of an iron complex of the phenol, for it was observed that the same colour could be obtained when $FeCl_3$ was replaced by the oxidizing agents, potassium permanganate, faintly acid sodium dichromate and chloramine T. On oxidation 3-hydroxysulphanilamide should give rise to a quinone imine thus:



Quinone imines, however, are not highly coloured compounds (see Sidgwick, 1942), and it seemed unlikely that the colour could be due to such a compound. The usual way of preparing quinone imines is to oxidize the aminophenol in ethereal solution with silver oxide. A saturated ethereal solution of 3-hydroxysulphanilamide (sparingly soluble in ether) was therefore shaken with Ag_2O at room temperature. After a few minutes the solution had a reddish tinge, and after half an hour the solution had become an intense red (containing some blue) showing an absorption band in the visible with $\lambda_{max.}$ at 532 mμ. (see Fig. 2). In the ultra-violet the solution showed two bands with maxima at 261·5 and 298 mμ. due to 3-hydroxysulphanilamide. The absorption spectrum of 3-hydroxysulphanilamide freshly dissolved in water shows two bands, $\lambda_{max.}$ 259 and 292 mμ. (see Fig. 3). On evaporation of the red ethereal solution to dryness a small amount of a brownish residue was obtained which, when dissolved in water, showed absorption maxima at 257, 291 and 440 mμ., the last one being of low intensity (Fig. 2). The first two are due to 3-hydroxysulphanilamide. The third one was found present in the absorption spectrum of an aqueous solution of 3-hydroxysulphanilamide which had been allowed to stand for a week at room temperature in the dark ($\lambda_{max.}$, 259, 292 and 442 mμ.; see Fig. 3). The solution at this stage was brownish yellow in colour. It appears probable that in aqueous solution 3-hydroxysulphanilamide is partly transformed into a quinone imine which then

polymerizes to a brownish substance (cf. Sidgwick, 1942) which is responsible for the band at about 440 mμ. It should be noted that the brown material obtained by evaporation of the red ethereal solution, does not give a red solution on redissolving in ether.

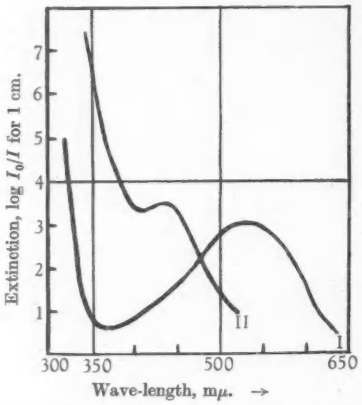


Fig. 2. I, absorption spectrum of the red solution obtained by treating 3-hydroxysulphanilamide in ether with silver oxide. II, absorption spectrum of an aqueous solution of the brown residue obtained on evaporation to dryness of the red solution I.

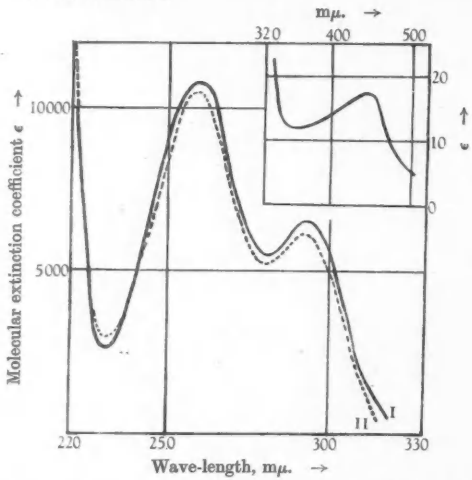


Fig. 3. Absorption spectrum of 3-hydroxysulphanilamide in water. I, fresh solution, $\lambda_{max.}$ 259 and 292 with log $\epsilon_{max.}$ 4·03 and 3·82. II, and inset, after standing a week, $\lambda_{max.}$ 258·6, 292 and 442 with log $\epsilon_{max.}$ 4·02, 3·78 and 1·24.

The above observations lead to the conclusion that the red ethereal solution may be due to the formation of a semiquinone type of compound which is stabilized in some way in ether. It was found that ether could be replaced by certain other solvents such as ethyl acetate, methyl ethyl ketone, *n*-butanol, amyl alcohol, but not by benzene, toluene, light petroleum, carbon tetrachloride or chloroform. The solvents in the former group are compounds containing 'lone pairs' of electrons, and stabilization of the semiquinone type of compound by co-ordination with the solvent is therefore conceivable.

These conclusions may be summarized as follows:

3-hydroxysulphanilamide (no band in the visible) → semiquinone → quinone imine

semiquinone → (suitable solvent) → red compound ($\lambda_{max.}$ at 530 mμ.)

quinone imine → polymerization product ($\lambda_{max.}$ at 440 mμ.)

Both maxima, 530 and 440 mμ., can be used for the characterization of 3-hydroxysulphanilamide.

A similar investigation was carried out on the closely related 4-hydroxymetanilamide (4-hydroxy-3-aminobenzenesulphonamide) which is a metabolite of metanilamide (Dobson & Williams, 1946). Although an aqueous solution of this compound gives with $FeCl_3$ practically the same colours as does 3-hydroxysulphanilamide (3-hydroxy-4-aminobenzenesulphonamide), it does not give rise to a red colour in ether for the intermediate semiquinone stage is apparently not detectable and the brown stage appears immediately in ether. The brownish polymerization product from this phenol shows a band with a maximum at 418 mμ. This band develops very readily when an ethanolic solution of the phenol is allowed to stand (see Fig. 4). The solution turns brown on standing.

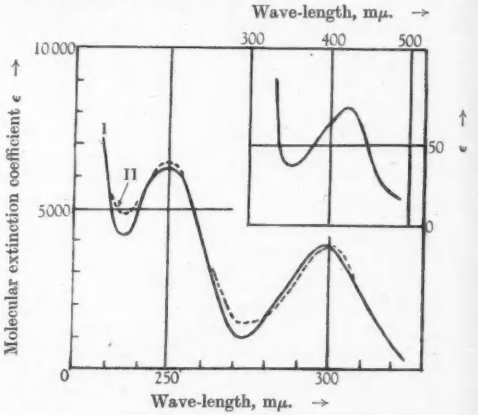


Fig. 4. Absorption spectrum of 4-hydroxymetanilamide in ethanol. I, fresh solution, $\lambda_{max.}$ 249 and 299 with log $\epsilon_{max.}$ 3·8 and 3·6 respectively. II, and inset, after standing 3 weeks, $\lambda_{max.}$ 249, 300·5 and 418 with log $\epsilon_{max.}$ 3·81, 3·59 and 1·53 respectively.

The other possible metabolites of sulphanilamide, which give coloured solutions with ferric chloride, are *p*-*N*-acetylhydroxylaminobenzenesulphonamide, 2-hydroxysulphanilamide and *p*-aminophenol. Of these only *p*-aminophenol gives the ferric chloride reaction in ether, and in this case the colour obtained is red (with no blue) showing an absorption maximum at 510 mμ. (see Fig. 1). This colour is much

weaker than the one obtained with 3-hydroxysulphanilamide, and it is clear from Fig. 1 that if *p*-aminophenol were present with 3-hydroxysulphanilamide it could not be detected. However, *p*-aminophenol can be eliminated if one takes into account the brownish polymerization product of oxidized *p*-aminophenol. This product shows a band at 395 mμ. which is easily distinguishable from the 440 mμ. band of the polymer of oxidized 3-hydroxysulphanilamide.

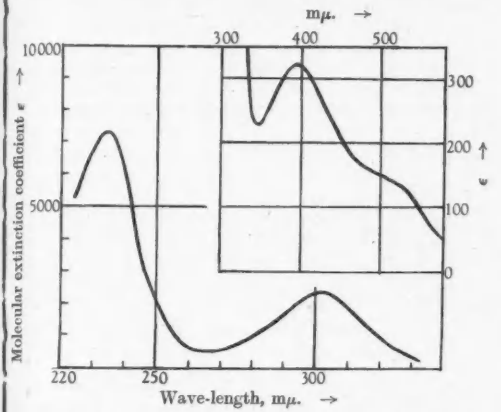


Fig. 5. Absorption spectrum of oxidized *p*-aminophenol in ethanol $\lambda_{max.}$ 234, 302 and 395 with log $\epsilon_{max.}$ 3·86, 3·38 and 2·51 respectively.

The 395 mμ. band was never encountered in the spectra of any of the urine extracts examined. *p*-Aminophenol (British Drug Houses Ltd.) as taken from the bottle is a black crystalline solid, and its absorption spectrum shows maxima at 234, 302 and 395 mμ. (see Fig. 5). This indicates that ordinary commercial *p*-aminophenol is already in a partly oxidized state, for earlier determinations of the spectral absorption of *p*-aminophenol only record the first two maxima (cf. Morton & Stubbs, 1940).

The colour reactions of the above-mentioned compounds are summarized in Table 1, which also includes salicylic acid since it was encountered in one of the human urines examined.

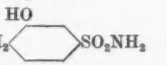
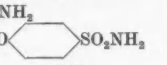
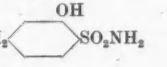
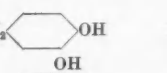
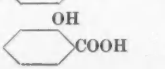
RESULTS

The detection of 3-hydroxysulphanilamide in the urine of hospital patients

(1) *Patient P.M.* The urine (3 l.), collected during a course of 26 g. of sulphanilamide, was taken to dryness under reduced pressure. The residue was dissolved in 100 ml. hot water and treated with 50 ml. conc. HCl. The mixture was boiled gently for 2 hr. After cooling the solution was made faintly acid to litmus with 20% (w/v) NaOH and, when cold, filtered. The filtrate was extracted with ether for 5 days in a continuous extractor. The extract was evaporated to a brownish mass of syrupy crystals (7 g.) and as much sulphanilamide as possible was removed by recrystallization from water. 4·3 g. of sulphanilamide, m.p. 162°, were obtained. The filtrate was dried *in vacuo* to a syrupy residue (2·6 g.) which gave colour tests for 3-hydroxysulphanilamide although containing much sulphanilamide. These were separated by chromatography.

The syrupy residue was dissolved in 50 ml. ethanol and 150 ml. dry benzene added. The solution was then chromatographed on an alumina column (6 × $\frac{3}{4}$ in.; aluminium oxide, standardized according to Brockmann; Savory and Moore Ltd., London) which was then eluted by ethanol until the eluates gave no diazo test. The eluates yielded 1·8 g. of sulphanilamide. The 3-hydroxysulphanilamide remained on the column forming a yellow band. The latter was separated from the column and exhaustively extracted with hot water. The extract was evaporated under reduced pressure to a clear yellowish glass (0·6 g.) which could not be induced to crystallize. It gave all the qualitative tests

Table 1. *The colour reaction between certain phenols and ferric chloride in water and in ether*

Compound	Colour produced in: Water	Colour produced in: Ether	$\lambda_{max.}$ of the absorption band of coloured ethereal solution (mμ.)
	Purplish red → red → brown	Intense red (containing some blue)	530
	Do.	Pale yellow	—
	Faint purple-red	None	—
	Blood-red	None	—
	Purple-red	Red (no blue)	510
	Purple	None	—

for 3-hydroxysulphanilamide (Williams, 1946). For the spectroscopic test, 0·3 g. of the glass was dissolved in 50 ml. water and then 1 ml. of 2% $FeCl_3$ followed by 50 ml. peroxide-free ether were added. The mixture was shaken and the red ether layer separated and filtered. The absorption spectrum of the ethereal solution showed a band with a maximum at 530 mμ.

(2) *Patient J.H.* The urine (3 l.) was collected during a course of 24·5 g. of the drug. It was concentrated, hydrolyzed and extracted as in the first case. The weight of material extracted in 7 days was 9·83 g., and this yielded 6·1 g. of sulphanilamide leaving a residue of 2·35 g. for chromatography. The yellow band on the column was prepared and extracted as in the first case, and the spectrogram (Hilger E3 Quartz Spectroscope) of the ether/$FeCl_3$ test is recorded in Fig. 1.

(3) *Patient J.K.* The urine (6·32 l.) was collected during a course of 44 g. of sulphanilamide with sodium salicylate. The urine was treated as above, and the material extracted by ether weighed 15·8 g. and contained salicylic acid. Sulphanilamide and some salicylic acid were removed by crystallization from water. The residue (4 g.) for chromatography contained 3-hydroxysulphanilamide, sulphanilamide and salicylic acid. These were separated as follows:

The residue was dissolved in a mixture of 50 ml. ethanol and 50 ml. dry benzene. This solution was passed through an alumina column (35 × 1 cm.) which was eluted with ethanol until both sulphanilamide and its phenol had been removed. The salicylic acid remaining on the column was extracted with hot water and purified (m.p. and mixed m.p. 153–155°) by recrystallization from acidulated water.

The eluate containing sulphanilamide and the phenol was now taken to dryness and the residue (1·5 g.) dissolved in 25 ml. ethanol and 75 ml. dry benzene. The solution was chromatographed on alumina (column, 20 × 2 cm.) and the sulphanilamide washed through with ethanol. 3-Hydroxysulphanilamide remained on the column as a yellow band. Part of this band was used for the colour test with positive results. Attempts to isolate the crystalline phenol from the rest of the band failed.

3-*Hydroxysulphanilamide in rabbit urine*

The colour reaction in this case was carried out on the urine of sulphanilamide-fed rabbits from which pure crystalline 3-hydroxysulphanilamide had been separated by chromatography (see Williams, 1946). The spectral absorption of the urinary product was determined with the following results:

	Urinary 3-hydroxysulphanilamide		Synthetic 3-hydroxysulphanilamide	
$\lambda_{max.}$ (mμ.)	259	291	259	292
log $\epsilon_{max.}$	4·04	3·81	4·03	3·82

A portion of the alumina column from which the crystalline phenol was isolated was extracted with water as described above for the human urines. The ether/$FeCl_3$ test was carried out on this extract. The ether solution had a deep red colour, and its spectral absorption is recorded in Fig. 1. In this case a weak band with $\lambda_{max.}$ 440 mμ. was found as well as the band at 530 mμ. Here evidently the oxidation had proceeded to some extent beyond the red pigment stage to form some of the brownish material with a band at 440 mμ.

Detection of 3-hydroxysulphanilamide in guinea-pig urine

Six guinea-pigs were kept for a week on a diet of crushed Lever's cubes containing 1% added sulphanilamide, and cabbage. This diet was only partly eaten. The week's urine (710 ml.) was analyzed for free and total sulphanilamide by the Bratton & Marshall method and was found to contain 1·98 g. total and 1·01 g. free sulphanilamide. The urine was worked up by the procedure used for human urines and very small amounts of 3-hydroxysulphanilamide were detected.

PREPARATION OF COMPOUNDS

3-Hydroxysulphanilamide (m.p. 164°). Some improvements were made in the synthesis of Thorpe & Williams (1941). Benzoxazolone was previously prepared in poor yields from salicylamide according to Graebe & Rostovzeff (1902). 50% yields of this compound have been recorded by Scudi & Buhs (1941) using *o*-aminophenol and phosgene. It has now been found that this compound can be prepared in yields of over 75% by simply heating *o*-aminophenol with urea.

o-Aminophenol (50 g.) and urea (50 g.) were heated together on an oil-bath at 180° (bath temp.) until the evolution of ammonia had ceased (*c.* 1–1·5 hr.). The cooled solid product was recrystallized, and decolorized with charcoal, from a large volume of hot water. The yield of pure benzoxazolone, m.p. 140–141°, was 47 g. (76% of calc.).

Benzoxazolone was converted into 2-oxybenzoxazole-6-sulphonyl chloride according to Scudi & Buhs (1941) and then into the amide. Benzoxazolone sulphonamide was converted into 3-hydroxysulphanilamide by gentle heating with 20% (w/v) NaOH. On cooling and neutralizing with HCl, the phenol crystallized out (cf. Thorpe & Williams, 1941). It was recrystallized from water.

2-Hydroxysulphanilamide (m.p. 154°) was a sample prepared by Thorpe & Williams (1941).

4-Hydroxymetanilamide (m.p. 202°) was prepared essentially according to Kermack, Spragg & Tebrich (1939) (cf. Williams, 1942).

p-N-*Acetylhydroxylaminobenzenesulphonamide* (N^4-acetyl-N^4-hydroxysulphanilamide), m.p. 223°, was prepared from *p*-hydroxylaminobenzenesulphonamide (1 mol.) and acetic anhydride (1 mol.) according to Burton & Walker (1943).

It was found that if an excess of acetic anhydride were used the product was p-O:N-*diacetylhydroxylaminobenzenesulphonamide* (N^4-acetyl-N^4-acetoxysulphanilamide). This diacetyl compound appears to be new. On recrystallization from water it forms large faintly yellow prisms, m.p. 171°. (Found: C, 44·2; H, 4·55; N, 10·3; S, 11·4. $C_{10}H_{12}N_2O_5S$ requires C, 44·1; H, 4·4; N, 10·3; S, 11·8%.)

p-Aminophenol (British Drug Houses Ltd.) was converted into the hydrochloride and used as this salt, thus ensuring that it was largely in the reduced form.

DISCUSSION

With the isolation of crystalline 3-hydroxysulphanilamide from rabbit urine (Williams, 1946) and its detection in human urine by the colour reactions described above, it is felt that a reasonable proof as to the exact chemical nature of the biological oxidation product of sulphanilamide has been provided.

These results have an important bearing upon certain theories of the mode of action of sulphanilamide. Early theories of sulphonamide action, such as those of Mayer & Oechslin (1937) and of Locke, Main & Mellon (1938), postulated that the antibacterial activity of sulphanilamide was due to a metabolic oxidation product, and *p*-hydroxylaminobenzenesulphonamide was considered to be the most likely compound to fill this role. The correctness or otherwise of these theories, however, cannot be proved until the exact chemical nature and the antibacterial properties of the oxidation product have been established. The case against *p*-hydroxylaminobenzenesulphonamide has been considered in an earlier paper (Williams, 1946) and need not be discussed further.

As 3-hydroxysulphanilamide is undoubtedly the oxidation product involved, its antibacterial properties have been tested *in vivo* and *in vitro* (see Appendixes 1 and 2). *In vivo* it has no therapeutic action against haemolytic streptococci in mice, but *in vitro* it shows definite antibacterial activity although of a lesser order than sulphanilamide. Its *in vivo* inactivity may be due to too rapid excretion and/or conjugation to form an inactive glucuronide (Williams, 1943) and an ethereal sulphate. Its slight activity *in vitro* is not unexpected for a number of substituted *o*-aminophenols have been found to be antibacterial *in vitro* (Barber & Haslewood, 1944, 1945; Martin & Rose, 1945). Its *in vitro* activity is, however, different from that of sulphanilamide for it is less affected by the numbers of bacteria than sulphanilamide. A possible explanation for this is that on standing in solution it is partly converted to the quinonoid form which may be more active than the reduced form.

The activity of sulphanilamide is unlikely to be due to an oxidation product for the following reasons:

(*a*) The amount of oxidation product, i.e. 3-hydroxysulphanilamide, produced is small and is not more than 10% of the dose of sulphanilamide (cf. Shelswell & Williams, 1940).

(*b*) The antibacterial activity of 3-hydroxysulphanilamide when tested *in vitro* is less than that of sulphanilamide.

(*c*) When tested *in vivo*, 3-hydroxysulphanilamide shows no therapeutic activity.

It is felt, therefore, that these results dispose of those theories which postulate that the activity of sulphanilamide is due to oxidation products.

It is well known that treatment with sulphanilamide often produces cyanosis, and methaemoglobin and occasionally sulphaemoglobin can be detected in the blood. It is possible that 3-hydroxysulphanilamide is involved here, for it can set up a reversible oxidation-reduction system

aminophenol $\rightleftharpoons$ quinone imine

which could result in the conversion of haemoglobin into methaemoglobin or into sulphaemoglobin if sulphide is present. *In vitro*, however, 3-hydroxysulphanilamide does not convert haemoglobin to methaemoglobin.

SUMMARY

1. A colour test is described for the detection of 3-hydroxysulphanilamide in urine. The theoretical basis of the test is discussed.

2. By means of this test 3-hydroxysulphanilamide has been detected in the urine of hospital patients treated with sulphanilamide and also in the urine of rabbits and guinea-pigs similarly treated.

3. The absorption spectra of 3-hydroxysulphanilamide, 4-hydroxymetanilamide and *p*-aminophenol have been recorded and discussed in relation to the colour test developed.

4. The bearing of the above results, together with those quoted in the Appendixes to this paper, on certain theories of sulphanilamide action is discussed.

The author wishes to thank Prof. R. A. Morton for assistance with the spectroscopic part of the work, Drs A. R. Martin (Imperial Chemical Industries Ltd.) and M. G. McEntegart for testing 3-hydroxysulphanilamide for antibacterial activity and Dr I. Ansell for the human sulphanilamide urines. The expenses of the work were in part defrayed by a grant from the Medical Research Council.

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Appendix 1. The *in vivo* Antibacterial Activity of 3-Hydroxysulphanilamide

3-Hydroxysulphanilamide was tested *in vivo* for antibacterial activity by Dr A. R. Martin. The following experiment, quoted from Dr Martin's report, shows that 3-hydroxysulphanilamide gives no protection to mice infected with *Streptococcus pyogenes*.

Groups of six mice were infected intraperitoneally with 0·2 ml. of a 1 : 100,000 dilution of an 18 hr. culture of *Streptococcus pyogenes* in blood-broth. The drugs used were prepared as dispersions and were given subcutaneously or intraperitoneally. Two doses were given daily, in the morning and in the evening, the first dose being given shortly before infection. Treatment was continued for 3 days where the mice were still alive. The results of one experiment are quoted in Table 2:

Table 2. *Treatment of an experimental streptococcal infection in mice with sulphanilamide and 3-hydroxysulphanilamide*

Treatment	Dose (mg./20 g.)	No. of deaths on: Day of infection	1st day	2nd day	Total deaths	Mean* survival time
None (controls)	—	—	4	2	6	0·8
Sulphanilamide, twice daily, subcutaneously	10	—	—	5	5	2·0
	5	—	—	6	6	1·0
	2	—	5	1	6	0·8
3-Hydroxysulphanilamide, twice daily, subcutaneously	10	—	6	—	6	0·8
	5	—	5	1	6	0·8
	2	—	6	—	6	0·8

* Calculated according to Whitby (1938).

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Appendix 2. The *in vitro* Bacteriostatic Activity of 3-Hydroxysulphanilamide

By M. G. McENTEGART, *Department of Bacteriology, University of Liverpool*

Pure synthetic 3-hydroxysulphanilamide was tested for bacteriostatic activity in parallel with sulphanilamide. In the preliminary tests, use was made of 'ditch plates' using 5·0 % lysed horse-blood agar to overcome any possible sulphanilamide inhibiting effect of the agar (Harper & Cawston, 1945). Plates were prepared with increasing dilutions of the test substances and were inoculated with streaks, at right angles to the ditch, of a 24 hr. culture of a known sensitive haemolytic streptococcus and of 10-fold dilutions of it—7 streaks being made in all. It was observed that in effective concentrations, 3-hydroxysulphanilamide produced inhibition irrespective of the size of the inoculum whereas

Table 3. In vitro *bacteriostatic activity of 3-hydroxysulphanilamide*

Agar ditch method.
Organism used: β-haemolytic streptococcus.
Inoculum: 1 in 10,000 dilution of a 24 hr. broth culture.

Compound	Concentration in agar ditch (%)	Distance from edge of ditch for which bacterial growth was inhibited (mm.)
3-Hydroxy-sulphanilamide	0·33	15
	0·10	10
	0·03	0 (no inhibition)
Sulphanilamide	0·33	20–30
	0·10	25
	0·03	25

sulphanilamide did not produce inhibition of the undiluted inoculum even in concentrations of 1·0%. The results in Table 3 were obtained with a 1 : 10,000 dilution of the 24 hr. culture of the test organism as inoculum.

The two compounds were tested for activity against a haemolytic streptococcus in the presence of blood using the slide-cell method (Fleming, 1944) with the following results (see Table 4) after 24 hr. (inoculum approximately 100 bacteria per cell).

Comment

The results of the above observations show, that by the three methods of testing—in agar, in peptone water and in blood—the bacteriostatic concentration effective against a haemolytic streptococcus of 3-hydroxysulphanilamide is 4–10 times that of sulphanilamide when a small number of bacteria is used as inoculum. When *S. aureus* is used as the test organism in broth, 3-hydroxysulphanilamide was at least as active as sulphanilamide (Table 5).

Apart from this difference in relative activities of the two compounds against haemolytic streptococci and *S. aureus* the range of bacteriostatic activity against different species of bacteria seemed to be the same for the two compounds.

Whereas the activity of 3-hydroxysulphanilamide was generally less than that of sulphanilamide, the size of the inoculum of haemolytic streptococci

Table 4. In vitro *bacteriostatic activity of 3-hydroxysulphanilamide*

Slide-cell method.
Inoculum size: approx. 100 bacteria per cell.
Organism used: β-haemolytic streptococcus.

Compound	Concentration, 1 in								
	400	800	1600	3200	6400	12,800	25,600	51,200	124,000
3-Hydroxysulphanilamide	–	–	+	+	+	+	+	+	+
Sulphanilamide	–	–	–	–	–	–	–	+	+

+ indicates growth of streptococcus after 24 hr.
– indicates no growth of streptococcus after 24 hr.

Further tests were carried out with serial dilutions of the compound in glucose peptone water against a haemolytic streptococcus and the Oxford *Staphylococcus aureus*. These tests were put up in duplicate with different numbers of bacteria in the inoculum (Table 5).

A *S. aureus* strain which was resistant to penicillin gave results similar to those with Oxford staphylococcus. Strains of *E. coli*, *Ps. pyocyanea* and *B. subtilis* were found to be insensitive to either compound.

Table 5. In vitro *bacteriostatic activity of 3-hydroxysulphanilamide*

Serial dilution method.
Organisms used: β-haemolytic streptococcus and *S. aureus*.
Inoculum size: approx. 10,000 and 100 organisms.

Compound	Organism	Approx. no. of bacteria	Observation made after hr.	Concentration of drug, 1 in						
				400	800	1600	3200	6400	12,800	25,600
3-Hydroxysulphanilamide	Haemolytic streptococcus	10,000	24	–	–	+	+	+	+	+
		10,000	48	–	–	+	+	+	+	+
		100	24	–	–	–	+	+	+	+
		100	48	–	–	+	+	+	+	+
	S. aureus	10,000	24	–	+	+	+	+	+	+
		10,000	48	–	+	+	+	+	+	+
		100	24	–	–	+	+	+	+	+
		100	48	–	+	+	+	+	+	+
Sulphanilamide	Haemolytic streptococcus	10,000	24	–	–	–	+	+	+	+
		10,000	48	+	+	+	+	+	+	+
		100	24	–	–	–	–	–	+	+
		100	48	–	–	–	–	+	+	+
	S. aureus	10,000	24	+	+	+	+	+	+	+
		10,000	48	+	+	+	+	+	+	+
		100	24	–	–	+	+	+	+	+
		100	48	+	+	+	+	+	+	+

+ indicates bacterial growth. – indicates no bacterial growth.

affected the results with sulphanilamide. While increase in the inoculum within the range tested did not affect the action of 3-hydroxysulphanilamide it had a marked effect in reducing the action of sulphanilamide both on solid and liquid media (Table 5).

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The Toxicity of Arsine Solutions for Tissue Slices

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(*Received* 12 *July* 1946)

When arsine enters the blood stream from the lungs, it is rapidly fixed by the red cells. After a short latent period, haemolysis occurs. It has been generally held that no arsine entering the body can escape reaction with the erythrocytes, and that poisoning of vital organs is thus due entirely to the substances liberated from the red cells on lysis. In recently published work, Levvy (1946), however, has shown that the lethal dose of arsine for mice, as measured by the arsenic absorbed in exposures to the gas causing 50 % mortality, which varies inversely with the gas concentration, can be as little as 0·67 mg./kg. At this value for the median lethal dose, arsine is much more toxic than arsenites, the most toxic arsenic derivatives likely to be formed in the reaction between the gas and the erythrocytes.

In the same paper (Levvy, 1946) experiments were described in which the arsenic content was determined of tissues from rabbits killed immediately after short exposures to arsine. Although the tissues were freed from blood before the onset of haemolysis, certain organs were found to contain considerable amounts of arsenic. Liver had the highest arsenic content, while kidney and lung also contained comparatively large quantities. It was concluded that this arsenic represented a fraction of the arsine which had been removed from physical solution in the plasma before it could be fixed by the red blood corpuscles. None of the arsenic fixed in this way is released until haemolysis commences (Thauer, 1934). Since it seemed possible that the effects of unchanged arsine on vital organs might explain, partly at least, the high toxicity of the gas for the whole animal, it was decided to determine the toxicity of arsine, in comparison with that of an arsenite, for surviving liver and kidney slices.

EXPERIMENTAL

To determine the toxicity of arsine for the tissue slices, it was not considered adequate to shake them in suspension in Ringer solution with a gas phase containing the arsenic derivative, since, to compare the effects with those produced by arsenite solutions, assumptions would have been required as to the distribution of arsine between the gas and liquid phases. A technique was accordingly developed for treating the surviving tissues with arsine solutions in absence of a gas phase, under conditions resembling as closely as possible those obtaining *in vivo*.

Method

Rat-liver or kidney slices were treated for exactly 20 min. at 37° with the arsine solution in a closed vessel completely filled with liquid. The solution was composed of a mixture of oxygenated Ringer solution (50–90 % of the total volume) and Ringer solution (10–50 % of the total volume) saturated with arsine in hydrogen. Other slices from the same animal were treated with either pure Ringer solution or with a solution of arsenite in Ringer solution, both of which contained quantities of oxygen and hydrogen similar to those present in the arsine vessel. Sufficient oxygen was always present to supply the tissue slices for 20 min. After this treatment, the oxygen consumption of the slices was measured by the Warburg technique (Dixon, 1934). Phosphate Ringer solution (Krebs, 1933) containing glucose (0·2 %) was used in all stages of the experiments.

Procedure

The gas towers. In all, four towers were required for saturating Ringer solutions with various gases. Each contained 200 ml. fluid. If these towers are numbered 1, 2, 3

and 4, their contents and the gases passed through them were as shown below.

Tower	Solution	Gas
1	Phosphate Ringer	O_2
2	Phosphate Ringer	$AsH_3 + H_2$
3	Phosphate Ringer	H_2
4	Arsenite in phosphate Ringer	H_2

The design of the arsine tower is shown at *A* in Fig. 1, and the other towers were similar, but without the attachments *B* and *C*. Arsine-hydrogen mixture was prepared by the action of zinc on an acidified arsenite solution in presence of copper catalyst, analytical reagents being used throughout, and the gas was collected in the gasometer previously described (Levvy, 1946). The concentration was usually of the order of 20–40 mg. AsH_3/100 ml. Before entering the tower, the gas was passed through 10 N-NaOH solution. Hydrogen was prepared from the same reagents without arsenite, and after washing with alkali was passed through towers 3 and 4, in that order. Oxygen was taken from a cylinder. The solution in tower 4 was prepared by diluting a measured volume (usually 2 ml.) of 0·1 M-sodium arsenite (made from A.R. As_2O_3) to 200 ml. with Ringer solution.

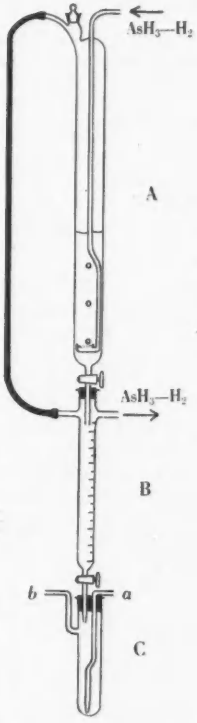

Fig. 1. Tower for saturating solutions with arsine (*A*), with attachments for measuring out (*B*) and collecting (*C*) samples for analysis.

The gas was passed through each tower for 10 min. at a rate of 50 ml./min. and then for 2–3 hr. at 5 ml./min. before using the solutions. Complete saturation of the solution in tower 2 with respect to the pressure of arsine present required about 3 hr., but latterly we did not always wait for equilibrium to be attained, as samples of the solution were withdrawn for analysis immediately before treating the tissues. While liquid was being withdrawn from a tower, the gas exit tube was clipped to prevent entrance of air.

Sampling the solutions. To determine its arsine content, the solution in tower 2 was measured out by means of the burette attachment *B* (Fig. 1), through which the arsine-hydrogen mixture was led after leaving the tower. After the burette had been washed out several times with the solution, a volume of about 5 ml. was run into the sampling tube *C* (Fig. 1). A slow stream of nitrogen was passed for 30 min. through *C* into a wash-bottle containing 10 N-NaOH and three absorption tubes charged with 0·02 N-$AgNO_3$ solution.

In early experiments, the flow of nitrogen was commenced before the arsine solution was measured out, and *C* remained attached to *B* throughout its passage. Latterly, it was found more convenient to detach *C* from *B* before passing nitrogen through the sample. The arsine solution was run into *C* with the nitrogen inlet and outlet tubes (*a* and *b*) closed by clipped rubber tubing, and *C*, complete with rubber stopper, was disconnected from the burette jet, the hole in the stopper being at once closed with a glass rod. This operation was easily performed if the burette tip and glass rod had been lubricated with glycerine. While arsine solution was entering, the pressure in *C* was released when necessary by opening the spring clip on the inlet (*a*) momentarily. The presence of a few drops of water in *C* acted as a trap for arsine in the inlet tube. This sampling procedure was found to yield as accurate and reliable results as that first used.

The arsenite formed in the $AgNO_3$ absorption tubes was titrated, after the addition of excess KI, with iodine in the presence of starch and bicarbonate. After all arsine had been removed with nitrogen, the solutions in the sampling tube *C* were examined for the presence of arsenite, invariably with negative results.

The arsenite content of the solution in tower 4 was, of course, known from its preparation. Titration with iodine showed no change in the arsenite content after passage of hydrogen for several hours.

Treatment of the tissue slices. The design of the vessels used for treating tissue slices with solutions from the gas towers is shown in Fig. 2. Before use, the capacity of each vessel was determined and it was then graduated in tenths of its total volume (60–70 ml.). For mixing, each vessel contained a hollow glass ball perforated at several points. Complete mixing of a coloured with a colourless solution could be achieved by dropping this ball the length of the vessel two or three times, but probably satisfactory mixing could be effected by movement of the tissue slices alone. The top of the vessel and the stopcocks were secured by elastic bands. Three vessels were required, one for treating the tissue with arsine, the second for arsenite treatment and the third for the control slices.

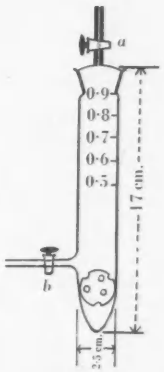

Fig. 2. Vessel for treatment of tissue slices in absence of a gas phase.

For treatment with arsine, tissue slices were placed in 2 or 3 ml. phosphate Ringer solution in the bottom of one of the vessels by means of long-handled forceps. The ball was then introduced and the top of the vessel attached. Oxygen was passed through the vessel till all air was displaced. With the capillary stopcock (*a*) open, oxygenated Ringer solution from tower 1 was run in through the wide-bore stopcock (*b*) till the solution reached the mark necessary for the desired dilution of the solution from tower 2, the vessel being always filled at least to the 0·5 mark with oxygenated Ringer solution. After samples of the arsine solution had been taken for analysis from tower 2, the burette was disconnected, stoppered and put aside. By means of a short piece of rubber tubing, well washed and

filled with solution from the tower, the arsine solution was run through (*b*) into the vessel till the liquid level just reached the capillary stopcock (*a*), care being taken to ensure that no bubbles were present before the vessel was completely filled. Both stopcocks were at once closed and the vessel inverted several times so as to mix the contents. Control and arsenite-treated slices went through the same process, the vessels being charged first with oxygenated Ringer solution and then with the solution in tower 3 or 4 respectively.

each experiment, and the figures for the Q_{O_2} (μl. oxygen absorbed in 1 hr./mg. dried tissue) are in most cases averages for duplicate determinations. Our own figures for the Q_{O_2} of normal rat tissues set up in Warburg flasks directly after slicing were kidney 25 and liver 7·6. It will be seen that treatment of the control slices for 20 min. with a solution of oxygen and hydrogen in absence of an oxygen gas phase does not seem to have greatly affected

Table 1. *Poisoning of tissue slices by arsine and by arsenite*

No. of exp.	Control slices Q_{O_2} (μl./mg./hr.)	Arsenite poisoned slices: Concentration (10^{-4} M)	Arsenite poisoned slices: Q_{O_2} (μl./mg./hr.)	Arsenite poisoned slices: Inhibition (%)	Arsine poisoned slices: Concentration (10^{-4} M)	Arsine poisoned slices: Q_{O_2} (μl./mg./hr.)	Arsine poisoned slices: Inhibition (%)
			Rat kidney				
1	23·1	5	14·4	38	5·0	13·2	43
2	19·0	—	—	—	5·6	10·8	43
3	19·9	2	12·4	38	0·74	15·0	25
4	19·2	2	14·9	22	1·1	19·7	0
5	20·4	5	12·4	39	1·8	14·9	27
6	20·0	5	11·8	41	4·2	13·5	33
7	19·7	4	16·9	14	3·9	16·4	17
8	19·6	4	18·3	7	3·9	16·4	16
9	20·9	5	13·3	36	4·4	15·2	27
			Rat liver				
10	7·2	2	4·8	33	1·5	2·3	68
11	5·8	5	3·0	48	2·2	1·7	71
12	7·4	2	5·0	32	0·72	5·4	27
13	6·9	5	3·3	52	1·0	4·0	42
14	6·3	1	4·3	32	1·7	2·8	56

All three vessels were set up in stands and shaken in a bath at 37° for 20 min. At the end of this period, the vessels were emptied, and the tissue slices, after brief washing with Ringer solution, were transferred to Warburg cups containing glucose-Ringer solution. The oxygen uptake was then determined. As a rule, sufficient tissue was placed in each vessel to charge two Warburg cups, and thus allow duplicate determination of the oxygen uptake.

In running it into the mixing vessel, no arsine was lost from solution. This was proved by blowing the contents of charged vessels with nitrogen into conical flasks, from which the nitrogen was led into $AgNO_3$ solution. After passing nitrogen through the conical flasks for 30 min. the figure for arsenite in the $AgNO_3$ solution was found to be within 10% of that calculated for arsine introduced into the mixing vessel. Further, when the oxygen displaced as the solution was run into the mixing vessel was passed through 1·5 ml. 0·02 N-$AgNO_3$ solution, no darkening occurred, although 0·5 μg. AsH_3 could be readily detected in this way.

RESULTS

Figures obtained for the oxygen uptake of control and poisoned slices of rat liver and kidney are summarized in Table 1. The inhibition of liver oxygen uptake caused by different concentrations of arsine and arsenite is shown graphically in Fig. 3. Slices from the same animal were used throughout their aerobic metabolism, and in very few cases was there any falling off in the oxygen uptake of the poisoned or control slices during the 70 min. they were shaken in the Warburg apparatus.

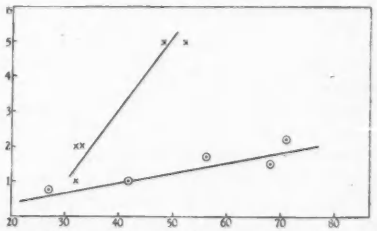

Fig. 3. Reduction of liver Q_{O_2} by arsine (⊙—⊙) and arsenite (×—×). Ordinate, concentration (10^{-4}M); abscissa, inhibition (%).

As regards the inhibition of oxygen uptake, arsine and arsenite were equally toxic for kidney slices (Table 1). In the case of liver, however, the molar concentration of arsine required for 50% inhibition in oxygen uptake was only one-quarter that of arsenite, as can be seen from Fig. 3. On treatment with arsine, but not with arsenite, liver slices underwent a marked colour change, assuming a greasy, yellow appearance.

DISCUSSION

Results obtained in previous work (Levvy, 1946) suggested that some arsine entering the blood stream may escape reaction with the red cells long enough to be fixed by the body tissues. From the experiments described above, it appears that if it does reach these organs *in vivo*, arsine will have a toxic action on the liver and kidneys. Under the conditions of the experiments, arsine was as toxic as arsenite for kidney tissue, and considerably more so for liver. The action of unchanged arsine in solution in the blood plasma on the liver, and perhaps also other organs, may thus explain, in part at least, the high toxicity of this gas when it is rapidly inhaled. The change in the appearance of liver slices when treated with arsine suggests that a reaction with the gas analogous to the characteristic one which occurs with haemoglobin may take place in the cells of this organ. An examination of the effects of poisoning *in vivo* with arsine on certain enzyme systems, besides yielding valuable information regarding the mode of action, might provide further evidence for the view that it has a specific action on body cells. It is of interest to note that arsenite, which did not change the appearance of liver slices, had the same toxicity for this tissue as for kidney.

If one accepts the postulate that the toxicity of arsine for the whole animal is in part due to effects produced by the gas itself in the body tissues, the decrease in its toxicity with decreasing concentration inhaled (Levvy, 1946) becomes readily explicable. As the rate of inhalation falls off, more of the gas will be fixed by the erythrocytes before it can be transported round the body, and thus transformed to less toxic arsenic derivatives. The nature of the arsenic-containing products of the reaction in blood has been studied by Graham, Crawford & Marrian (1946).

The technique described above may find general application in experiments in which it is desired to study the effect of known concentrations in solution of substances which are normally gases on the metabolism of surviving tissues.

SUMMARY

1. A technique was devised for treating surviving liver and kidney slices in absence of any gas phase with arsine solutions of known concentration.

2. The effect of arsine in reducing the oxygen uptake of rat kidney was identical to that of arsenite.

3. With liver, a 50% inhibition in the oxygen uptake was obtained with a lower concentration of arsine than of arsenite.

4. The bearing of these findings on the toxicity of arsine for the whole animal is discussed.

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Histidine Deficiency in the Rat and its Effect on the Carnosine and Anserine Content of Muscle

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The original purpose of the work described in this paper was to establish whether histidine was essential to the maintenance of body weight in the adolescent and adult rat and to investigate the symptoms of such deficiency if it occurred. Since our work has been completed, two papers dealing with the same subject have appeared. Albanese & Frankston (1945) have shown that histidine is required in the diet both by young and adult rats, if body weight is to be maintained. Maun, Cahill & Davis (1946) confirming this finding have noted a slight reduction in haemoglobin and plasma protein

content, a definite atrophy of the thymus and delayed spermatogenesis in histidine-deficient animals. Our results confirm fully those obtained by the American workers as far as loss of weight, anaemia and hypoproteinaemia are concerned and the slight differences observed may be mainly due to the longer period of histidine privation employed in many of our experiments.

One aspect of our work, however, has not been touched upon by the American workers and will be described in some detail. A deficiency of an essential amino-acid in the diet will in the first place cause a disturbance in the synthesis of protein in the body and so far as the function of an amino-acid is confined to that of building-material for protein synthesis, a dietary deficiency will produce unspecific symptoms which can only vary quantitatively with different amino-acids. But if one amino-acid has a function not shared by others, as, for example, of being the precursor of some particular body-constituent, specific changes either in the composition of tissues and/or in bodily functions may be expected to occur if such an amino-acid is omitted from the diet. It was shown by Schoenheimer, Rittenberg & Keston (1939) that whilst the amino-N of histidine was exchanged readily in the body with dietary $N^{15}H_3$, the nitrogen of the iminazole nucleus was quite inert. From this observation and the results reported in another paper (Neuberger & Webster, 1946) it can be concluded that the rat cannot synthesize this heterocyclic nucleus at all and it can be assumed that all body constituents possessing an iminazole nucleus are derived from dietary histidine. There are at least four substances besides histidine itself occurring in mammalian tissues which have an iminazole or a modified iminazole structure: carnosine, anserine, histamine and ergothioneine. There is considerable doubt as to whether ergothioneine is a normal body constituent and it is most probably entirely of exogenous origin (Potter & Franke, 1934). Histamine is present in minute quantities only and its estimation by either chemical or biological methods appeared to be of questionable value in view of the rather unspecific nature of the methods available. We have therefore directed our attention to the determination of the two β-alanine peptides of histidine and methylhistidine respectively, which occur in all mammalian muscles in considerable quantities and represent the largest store of histidine derivatives in the body apart from the even greater fraction of the amino-acid which is bound in proteins.

Carnosine is usually estimated by the Pauly reaction and, though this reaction is not very specific, it has been shown by Hunter (1922) that about 95% of the colour given by muscle extracts containing large amounts of carnosine, is in fact due to this peptide. Rat muscles contain very little carnosine, but relatively large quantities of anserine which have been identified by isolation (Wolff & Wilson, 1932). Anserine and *l*-methylhistidine do not give the Pauly reaction and no method for the direct estimation for either of these substances is available. Zapp & Wilson (1938) assumed that the sum of the two peptides is equivalent to the increase of α-amino-nitrogen obtained on hydrolyzing the material precipitable by mercuric acetate at neutral reaction. Carnosine was estimated by the diazo reaction and anserine by difference. The authors themselves have drawn attention to the possibility that other peptides precipitable by mercury may be present in muscle extracts and may give rise to a considerable error. Bate Smith (1939) suggested that the residual buffering power of rigor muscle, i.e. the difference between total buffering power and that due to protein, phosphate, carnosine and lactic acid, could give a 'rough guide to the amount of anserine present in muscle'. Neither of these two methods appeared to be sufficiently specific for our purpose, and the possibility of using the concentration of β-alanine as a measure of the combined anserine and carnosine content was therefore investigated. Several microbiological methods for the estimation of β-alanine have been described; the method used in our work was based on that described by Schenck & du Vigneaud (1944). In order to assess the suitability of this method for our particular purpose, it was necessary to investigate the distribution of β-alanine in different tissues. Pantothenic acid which also contains β-alanine has a wide distribution in animal tissues, and this and the possible presence of other so far unknown compounds containing β-alanine could invalidate this method as a measure of carnosine and anserine content of the body. Another point which had to be investigated was the interference with the assay method used by protein degradation products present in the hydrolysate.

EXPERIMENTAL

Litters of black and white rats of the Institute strain with initial weights varying in two separate series from 180–200 and from 93–111 g. respectively were used. Weights were recorded every second day and food consumption was also measured.

Diet. The dietary protein was given in the form of amino-acids and the composition of the amino-acid mixture and of the diet as a whole was identical with that described in an earlier paper (Neuberger & Webster, 1946). Controls were given in addition 35 mg. *l*-histidine/day. The intake of amino-acids in both groups was kept constant as far as possible and varied between 1·3 and 1·4 g. The intake of the non-protein part of the diet varied with the appetite. Animals on a 'non-protein' diet were not given amino-acids, but otherwise the same diet as above.

In order to investigate the effect of other variations in the diet small groups of animals were given 'casein',

tryptophan-deficient and stock diets respectively. The casein diet contained 16% of casein and the tryptophan-deficient diet contained 16% of zein supplemented with 50 mg. of *l*(+)-lysine hydrochloride/day. In these experiments the protein intake varied with the food intake. The other components of the diet were given in the same proportions as in the earlier experiments.

Haemoglobin and protein content of plasma and liver. The methods were the same as those used in an investigation on lysine deficiency (Gillespie, Neuberger & Webster, 1945).

Estimation of β-alanine. β-Alanine estimations were carried out both on hydrolysates of whole animals or separate organs and on deproteinized extracts of muscles and other tissues. For the hydrolysis of whole rats the directions of Schenck & du Vigneaud (1944) were followed. Muscle extracts were prepared by extracting ground muscles three times with water at 70° for three periods of 20 min., the combined extracts were filtered through muslin, made up to a known volume after addition of one or two drops of 25% acetic acid and heated gently to boiling. The coagulated protein was filtered off. A sample of this solution was used for the estimation of carnosine. For the determination of β-alanine 2 vol. of the extract were mixed with 1 vol. of 37% HCl (w/w) and the solution was then heated for 2 hr. at 100°, taken to dryness *in vacuo*, the residue dissolved in water and made up to volume.

The β-alanine contents of other tissues were determined either on hydrolyzed whole organs or on deproteinized extracts. In the latter case whole organs were ground in the Potter-Elvehjem homogenizer, suspended in 20 vol. of water made just acid to methyl orange with acetic acid, and heated at 70° for 20 min. This extract was then filtered and hydrolyzed in the same manner as described for muscle. When the complete hydrolyzed tissue was desired, organs were cut up with scissors and boiled under reflux for 10 hr. after addition of 20 times their weight of 20% HCl (w/w). The further treatment followed exactly that described above for muscle.

Tissue extracts or hydrolysates were assayed for β-alanine by a yeast-growth method which was very similar to that used by Schenck & du Vigneaud (1944) using the Gebruder Mayer strain of *Saccharomyces cerevisiae* (National Type Collection No. 4614). This strain, however, cannot utilize carnosine instead of β-alanine, in contrast to the strain used by the American workers. It was also found that with the medium used by Schenck & du Vigneaud the slope of the growth curve was steeper with hydrolysates of rat tissues than with β-alanine solutions and this appeared to be due to the presence of growth factors other than β-alanine in the extracts. The addition of an acid hydrolysate of vitamin-free casein to a final concentration of 0·04% to the basal medium removed this discrepancy. Growth was estimated by measuring the turbidity of the yeast suspension with a Spekker absorptiometer with a neutral screen of 1·5 density. Growth was linearly proportional to the β-alanine concentration over a relatively narrow range and this part of the curve was fairly steep. Care was taken to dilute samples in such a way as to obtain readings well within the linear range of the standard curve.

Inhibition of yeast growth by other tissue components. In most of our experiments protein-free extracts of muscles were used; such extracts are rich in β-alanine and contain presumably only small amounts of other substances having either a stimulating or inhibitory effect on the growth of yeast. Recovery of added β-alanine with such extracts is quantitative within the limits of reproducibility of the method which is about 5%. When whole tissues, however, are hydrolyzed and particularly tissues containing only traces of β-alanine, considerable inhibition by products of hydrolysis occurs. This is shown by the fact that assay of whole hydrolyzed tissues such as lungs, kidney and blood indicated complete or almost complete absence of β-alanine, whilst protein-free extracts of these organs appeared to contain small, but measurable amounts of this amino-acid. This inhibition by tissue components has already been noted by Schenck & du Vigneaud (1944) in the case of liver and may be due to products of protein hydrolysis. Such an interpretation is supported by the work of Nielsen, Hartelius & Johansen (1944) who found that nearly all α-amino-acids counteract the stimulation of growth of *S. cerevisiae* produced by β-alanine. This inhibition becomes apparent when the ratio of α-amino-acid to β-alanine exceeds the value of 300. In our experiments the addition of a casein hydrolysate to both standard and test solutions makes allowance for this source of error and in protein-free extracts of muscle and liver, tissues which are rich in β-alanine, inhibition caused by α-amino-acids is not likely to be of importance. In hydrolysates of whole rats, the ratio of α-amino-acids to β-alanine is less favourable, but even in this case the amount of nitrogen added is smaller than that present in the basal medium and results can be considered fairly reliable, unless the β-alanine content of the hydrolysate is very low. Recovery of β-alanine added to hydrolysates of whole rats was quite satisfactory, but such a test is not very stringent. The addition of β-alanine to a hydrolysate increases the ratio of β-alanine to α-amino-acids and conditions are not strictly comparable to those obtaining in the hydrolysate itself. Evidence was in fact obtained indicating that the values of β-alanine found for whole rats may be too low by about 20%. Values obtained with hydrolysates of whole organs other than muscle and possibly liver are wholly unreliable.

There is of course the possibility, as in all microbiological assays applied to complex materials, that an inhibitory or stimulating effect on the growth of yeast may be exerted by other compounds present in the mixtures. We have no data which would definitely exclude such a possibility, though it appears unlikely in view of the good recovery of added β-alanine to these extracts. As will be shown later, the β-alanine values obtained for muscle are of the expected order if it is assumed that β-alanine is entirely derived from anserine and carnosine. Moreover, the β-alanine contents of whole rats agreed quite well with values calculated on the assumption that nearly the whole β-alanine of the body is contained in muscle. It is concluded therefore that the results obtained are reasonably reliable and are strictly comparable.

Estimation of carnosine. Carnosine was determined colorimetrically by the Pauly reaction with diazotized sulphanilic acid with carnosine nitrate as a standard. All the various published modifications of this method have been tried, but with rat muscles which contain only small amounts of carnosine a yellow colour was always obtained which must be due to a substance other than carnosine; this was particularly noticeable in extracts of very low carnosine content. In an attempt to overcome this difficulty which has been experienced by other workers (Eggleton & Eggleton, 1933) the method of Edlbacher, Baur, Staehelin & Zeller (1941) for the estimation of histidine was adapted to that of carnosine. In this method diazotized *p*-chloro-aniline

is used and the resulting pigment being soluble in butanol can be readily extracted from aqueous solution. It was found, however, that with rat muscle extracts the interfering pigment was also extracted into the organic solvent.

The procedure finally adopted was as follows. The cooled diazotized sulphanilic acid was added to the solution being tested and to the carnosine standard, the exact quantities and concentrations of reagents being similar to those used by Meschkowa (1936). After 10–15 min., when the colour had reached its maximum, the two solutions were compared in a colorimeter with a Wratten no. 75 filter. This, as pointed out by Eggleton & Eggleton (1933) makes matching easier, but does not necessarily avoid the error due to the substance giving the yellow colour. If about 5% of colour given by muscle extracts of horse or frog which are rich in carnosine is due to other substances (Hunter, 1922), this error must be considerably larger in rat muscles; it is believed therefore that the carnosine figures given in this paper are probably too high, but are nevertheless comparable.

RESULTS

Changes of body weight. Rats on a histidine-deficient diet cannot maintain their body weight (Table 1). The rate of loss is very marked in the first and second weeks of the deficiency, but becomes less pronounced in the later stages. The actual weight losses are smaller than those observed in deficiencies of some other essential amino-acids investigated by us such as methionine.

Haemoglobin content. Histidine deficiency leads to a mild anaemia as shown in Table 1 which indicates that the haemoglobin figures obtained for deficient animals are about 20–25% below those considered normal for rats of similar age. The anaemia found in histidine deficiency is similar to or slightly less severe than that observed in lysine deficiency (Gillespie *et al.* 1945).

Plasma protein content. Lack of histidine in the diet produces a marked reduction of plasma proteins, the average content after varying periods of deficiency being about 5·3%. The corresponding figure for lysine deficiency was found to be 5·2%, whilst in methionine deficiency in younger animals an average figure of 3·6% was obtained (Glynn, Himsworth & Neuberger, 1945). On a protein-free diet, values of 3·0–3·2% were obtained (Table 1).

Changes in the composition of the liver. Table 2 records the crude composition of the liver of animals which had been on a diet deficient in histidine for varying periods; corresponding figures are also given for animals on non-protein lysine-deficient diets, and

Table 1. *Changes of body weight, haemoglobin and plasma protein contents of rats receiving amino-acid diets with and without histidine and of rats on a non-protein diet*

(The letters A, B, etc. refer to litters; litter-mates were, as far as possible, evenly distributed over the different dietary groups. H indicates diet with histidine, HD, diet without histidine, and NP, non-protein diet.)

Rat no.	Body wt. Initial (g.)	Body wt. Final (g.)	Days on diet	Diet	Blood haemoglobin (g./100 ml.)	Plasma protein (%)	Average daily food consumption (g.)
B 1	157	113	54	HD	13·8	5·7	7·5
B 6	134	101	41	HD	12·4	—	6·4
C 1	207	156	64	HD	12·3	5·4	8·6
C 3	145	98	62	HD	—	—	6·5
C 4	140	92	63	HD	11·8	5·3	6·8
X 1	111	81	33	HD	13·0	5·0	5·2
Z 4	108	73	33	HD	12·4	5·0	5·5
Y 3	104	76	33	HD	13·0	5·0	5·2
Z 6	110	76	33	HD	13·3	4·8	5·4
B 2	150	233	54	H	16·5	7·0	14·0
A 1	93	55	42	NP	9·8	3·0	8·2
A 3	105	58	42	NP	11·5	3·2	8·4
Z 5	125	75	42	NP	10·2	3·2	5·2

Table 2. *Nitrogen, fat and water contents of livers of rats on different diets*

(All the figures, with the exception of those for liver water which are expressed in terms of mg./100 g. of body weight, refer to average values; the figures in brackets give the standard error of the mean.)

No. of animals used	Diet	Days on diet	Liver-N	Liver fat	Liver water
4	Histidine-deficient	33	114 (±5)	60 (± 7)	73·4 (±0·1)
5	Histidine-deficient	41–63	90 (±3)	56 (± 8)	73·6 (±0·2)
5	Non-protein	42	80 (±7)	197 (±25)	76·2 (±0·2)
5	Restricted stock diet	45	98 (±2)	65 (±15)	73·5 (±0·3)
7	Lysine-deficient	48	85 (±2)	160 (±35)	73·9 (±0·3)

of rats whose caloric intake was restricted in such a way as to keep their body weight about constant. The corrected water content, i.e. g. of water/100 g. fat-free liver was definitely elevated in the animals which had subsisted on a protein-free diet, was possibly slightly increased in lysine deficiency, but was definitely normal in histidine-deficient animals. The fat content of the livers was normal or even below normal in all the groups investigated. It is clear that diets deficient in the three essential amino-acids lysine, histidine or methionine (Himsworth & Glynn, 1945) do not produce fatty livers, if sufficient choline is given.

If a histidine-deficient diet is given for 33 days the total liver-N is only slightly reduced, i.e. on the average by about 10% (Table 2). Prolonged deficiency appears to lead to a much more severe reduction. The slight reduction in the first five weeks may be entirely due to the reduced caloric intake of the diet (Harrison & Long, 1945; Kosterlitz & Campbell, 1945).

The β-alanine content of tissues other than muscle. Table 3 shows the concentrations of β-alanine in several organs as found by ourselves in hydrolyzed protein-free extracts and also the amounts of pantothenic acid as observed by Mitchell & Isbell (1942). With the exception of muscle there is a close correlation between the two sets of values and it is highly probable that most of the β-alanine present in the body apart from that found in muscular tissue forms part of the pantothenic acid molecule. The total amount of β-alanine present in tissues other than muscle is comparatively small (Table 3); it can be estimated that at least 95% of the total β-alanine of the rat is present in muscle. Such an estimate is also borne out by a comparison of the β-alanine contents of isolated muscles and that of whole rats respectively.

Table 3. *The β-alanine contents of different organs of the rat*

(In these experiments the organs of three rats of a total wt. of 374 g. were pooled and treated as described in the text.)

Organ	Amount of tissue taken (g.)	β-Alanine found (mg.)	β-Alanine in tissue (mg./100 g.)	Pantothenic acid* in tissue (mg./100 g.)
Blood	9·2	0·017	0·19	—
Liver	19·1	1·43	7·5	7·3 –15·0
Kidney	3·4	0·034	1·0	3·2 – 3·4
Testis	6·2	0·028	0·45	—
Lung	3·0	0·010	0·35	0·85– 0·95
Heart	1·4	0·014	1·0	3·0 – 4·0
Gastro-intestinal tract	21·0	0·128	0·61	—
Brain	4·3	0·018	0·43	1·2
Combined muscles of hind-leg	25·0	15·25	61·0	0·45– 0·55
Combined muscles of shoulder	13·4	8·58	64·0	

* Figures given by Mitchell & Isbell (1942).

Table 4. *Carnosine and β-alanine contents of muscle extracts of rats on different diets*

Rat no.	Body wt. (g.)	Diet	Muscle content (mg./100 g.) of β-Alanine	Muscle content (mg./100 g.) of Carnosine
A 5	191	Stock diet	71	98
B 3	203	Stock diet	63	106
C 5	161	Stock diet	82	76
C 8	155	Stock diet	62	74
A 4	206	Casein	86	109
B 4	198	Casein	76	77
C 6	272	Casein	111	116
Z 1	180	Casein	70	69
B 2	233	Complete amino-acid	104	103
A 5	123	Non-protein	97	75
A 1	55	Non-protein	85	70
Z 5	75	Non-protein	105	95
B 7	132	Tryptophan-deficient	80	56
C 7	105	Tryptophan-deficient	85	61
Z 1	106	Restricted stock diet	80	77
Z 2	95	Restricted stock diet	91	84
B 1	113	Histidine-deficient	62	26
B 6	101	Histidine-deficient	65	29
C 1	125	Histidine-deficient	59	28
C 3	120	Histidine-deficient	63	31
X 1	81	Histidine-deficient	57	35
Z 4	73	Histidine-deficient	68	34

Table 4 shows the values for the mixed muscles of the fore- and hind-legs of rats on normal diets which varied between 55 and 115 mg. of β-alanine/100 g. of muscle with an average value of about 86 mg. If it is assumed that 95% of all the β-alanine is contained in muscle and that the muscles of the leg are representative of muscular tissue generally, then the whole β-alanine content of the body can be calculated, if the proportion of muscle relative to body weight is known. Estimates for this ratio made by different authors vary between 30 and 40%, and we may use for our calculation a value of 35%. The expected β-alanine content of the whole body on this basis is 28 mg./100 g. of body weight. The average value

Table 5. *β-Alanine contents of whole rats on different diets*

Rat no.	Wt.	Diet	Amount of β-alanine in rat (mg./100 g.)
D 1	110	Stock diet	19
E 3	200	Stock diet	31
D 3	55	Stock diet	24
D 4	98	Stock diet	23
B 1	113	Histidine-deficient	23
C 4	98	Histidine-deficient	30
Y 3	76	Histidine-deficient	20
Z 6	76	Histidine-deficient	20

found by Schenck & du Vigneaud (1944) and also by ourselves is about 23 mg.; the agreement is as good as can be expected in view of the many uncertainties involved.

The β-alanine content of muscle extracts. The amount of β-alanine in muscle extracts after mild hydrolysis is used in the present work as a measure of the concentration of the two peptides, carnosine and anserine. The assumption implied is that the amount of free β-alanine and the concentration of other compounds liberating β-alanine is negligible compared with that of carnosine and anserine. The validity of this assumption could not be tested directly, since no other reliable method for the estimation of β-alanine or for that of methylhistidine is at present available. But the following facts appear to justify this assumption. The amount of pantothenic acid in muscle is extremely small (Mitchell & Isbell, 1942) and only traces of free β-alanine could be found in fresh muscle extracts. No compound containing β-alanine other than carnosine, anserine and pantothenic acid has as yet been obtained from biological sources. Moreover, a comparison between the values of carnosine and anserine obtained by the assay of β-alanine and those of other workers who used different methods suggests that the method we have been using certainly does not over-estimate the concentration of these peptides. If the results of Table 4 are recalculated in terms of anserine the average amount in 100 g. of muscle is found to be about 230 mg. whilst the highest and lowest values are 310 and 140 mg. respectively. Zapp & Wilson (1933) found, with a rather unspecific method, a value of 360 mg./100 g. for the combined anserine and carnosine content of rat muscle, whilst Bate Smith (1939) calculated from the buffering curves of muscle extracts anserine contents varying between 160 and 310 mg./100 g. in good agreement with our figures.

β-Alanine and carnosine contents of muscles of rats and the effect of different diets. Table 4 shows that the β-alanine contents of muscles of rats supplied with a satisfactory diet (casein- and stock-diet groups) varied between rather wide limits. This agrees with the findings of other workers who studied the concentrations of carnosine in the muscles of species with low anserine and high carnosine contents (Hunter, 1924; Mitsuda, 1923; Eggleton & Eggleton, 1933). These authors found a wide variation in the figures obtained for the carnosine content of different muscles from the same animals and also for similarly placed muscles taken from different individuals of the same species. Removal of protein or of an essential amino-acid, like tryptophan, from the diet does not significantly affect the values, nor has restriction of caloric intake leading to loss of weight any apparent effect. The values for β-alanine found for muscles of rats which had been on a histidine-deficient diet for varying periods are near the lower limit of the normal range. It is doubtful, however, whether this slight apparent reduction is statistically significant in view of the wide variation in normal animals and the small number of data at our disposal. It can be concluded that at least in older animals prolonged dietary deficiency either does not lead to a change of the β-alanine and therefore of the combined carnosine and anserine content of muscle at all or leads only to a slight reduction which on the average does not exceed 25 %. The carnosine values appear to be definitely reduced in the histidine-deficient animals, but the quantitative significance of this finding is somewhat uncertain in view of the unspecific character of the method and the fact that carnosine has not to our knowledge been actually isolated from rat muscles.

It was found, however, that if animals are put on a histidine-deficient diet soon after weaning the β-alanine content decreases considerably to values of below 20 mg./100 g. and the substance(s) giving rise to the colour reaction with the diazo reagent disappear(s) almost completely from muscle extracts. It is doubtful if this finding can be attributed to histidine deficiency, since the weight of the animals is reduced to about 30 g. and rats of such low body weight usually have low β-alanine and carnosine values.

The effect of different diets on the β-alanine contents of whole rats. The data presented in Table 5 support the conclusions drawn from the β-alanine values of muscle extracts discussed above. The few normal values are within the range of the figures given by Schenck & du Vigneaud (1944) for a larger series of animals of similar weight. Deficiency of histidine in the diet has no significant effect on the β-alanine content of the whole body.

DISCUSSION

The essential character of histidine as a dietary constituent. The results reported in this and in an earlier paper (Neuberger & Webster, 1946), together with the observations of Albanese & Frankston (1945) and those of Maun *et al.* (1946), show clearly that neither the young nor adult rat can maintain its body weight if histidine is not present in the diet. Though loss of weight as such does not necessarily indicate loss of protein, a decrease amounting to a third or a fourth of the initial body weight, as was found in our experiments, must involve loss of body substance other than fat. It can be concluded that lack of histidine in the diet leads to loss of body-nitrogen and histidine must be classified as an amino-acid which is essential to the rat both for the purpose of growth and maintenance. The loss of weight is only partly due to the decreased food intake, since animals receiving a diet of a caloric value identical with that consumed by histidine-deficient animals

maintained their weight fairly well (Maun *et al.*, 1946). However, Burroughs, Burroughs & Mitchell (1940) found in experiments lasting 6–9 days that rats could maintain nitrogen equilibrium on diets not containing histidine, although some of their animals lost weight. A possible explanation for this discrepancy may be found in experiments on another species.

Dogs supplied by vein with all essential amino-acids remain in positive nitrogen balance (Elman, Davey & Loo, 1941). If histidine is omitted from the infusion fluid, the balance still remains positive for several days; after that the excretion of nitrogen in the urine rises and a marked negative nitrogen balance is observed. Similar results were obtained by Madden, Anderson, Donovan & Whipple (1945) on dogs whose plasma proteins had been depleted by frequent bleedings, the red cells being returned to the system. It was found that the ten essential amino-acids can cause abundant plasma protein production and maintain a positive nitrogen balance in these dogs. If histidine was omitted, there was at first no apparent change, but after a week the nitrogen balance became negative. What is the reason for this delay in the rise of nitrogen excretion? One possibility is, that the body can draw for a time on amino-acid reserves other than protein, to supply endogenous losses. Though the amounts of free amino-acids in plasma and tissues are low, they may be sufficient to replace, at least for a short time, an amino-acid which is irreversibly degraded at a slow rate only. Or it may be suggested as an alternative explanation that the body can still produce proteins for a time by internal rearrangement, if an amino-acid which cannot be synthesized, is omitted from the diet. Histidine for example is present in fairly large quantities in some proteins like haemoglobin, histones and insulin, whilst its concentration is smaller in most other proteins. It would be possible for the body therefore to break down histidine-rich proteins and to utilize the free amino-acid for the production of protein of low histidine content. In this way, or simply by differences in the rate of formation of proteins with different histidine content, a positive nitrogen balance could be maintained for a few days; only later, when the equilibrium between the different proteins becomes too greatly disturbed, will the body be unable to utilize other amino-acids and urinary excretion of nitrogen will increase. Whatever the explanation of this phenomenon may be, it is clear that the apparently contradictory results of Burroughs *et al.* (1940) on the one hand, and the findings of other American workers and those reported here on the other, are very similar to the observations made on histidine-deficient dogs.

In man, positive nitrogen balance can be maintained for weeks, though histidine is not present in the diet (Rose, Haines, Johnson & Warner, 1944). It follows from what has been said already that such a result does not necessarily indicate that man can maintain weight or nitrogen balance indefinitely on a histidine-free diet.

General symptoms of histidine deficiency. The loss of weight, anaemia, hypoproteinaemia and relative decrease of liver protein reported in this paper and the atrophy of the thymus, vascularization and epithelial metaplasia of the cornea found by Maun *et al.* (1946) in histidine-deficient rats present a picture which is very similar to that found in most deficiencies of amino-acids so far investigated in detail. The lack of an essential amino-acid in the diet always reduces food consumption, and loss of appetite with a resulting reduction of caloric intake is part of the effect of the dietary deficiency. The American workers have, however, shown that most of these changes are found either not at all or only to a lesser degree in rats receiving an equi-caloric diet and they must therefore be ascribed not to general malnutrition but to an inhibition of protein synthesis. The anaemia found was rather mild and this is particularly remarkable in view of the high concentration of histidine in haemoglobin. The histidine-deficient animals had a healthy appearance and, altogether, pathological changes were less marked than those found in deficiencies of other essential amino-acids. This could be explained by a limited synthesis of histidine by the rat. Such an explanation is unlikely for reasons discussed above. It appears more probable that these differences in the severity of symptoms are due to a quantitative variation in the demand for the different amino-acids, which in turn depends on the rate of irreversible degradation, and which may vary within wide limits for different compounds.

The carnosine and anserine contents of muscles of histidine-deficient rats. The function of these two peptides which are so universally distributed in muscles is still obscure although it has been suggested that they may be required for the regulation of pH (Bate Smith, 1938). It had been hoped to reduce the concentration of these substances by producing a dietary deficiency of their presumed precursor, histidine, and to relate any functional changes in the muscle to the decreased concentrations of the β-alanine peptides. It has not been possible to achieve this purpose. Even prolonged histidine deficiency leading to a loss of a considerable proportion of the protein of the body did not materially alter the concentration of β-alanine peptides either in the whole body or in particular muscles. It is likely that the formation of carnosine and anserine competes with other reactions requiring histidine, such as the formation of protein or histamine, and the net result of a dietary deficiency of histidine is a general and fairly uniform reduction in all these synthetic

processes leading to a loss of body substance as a whole without altering the relative composition of the tissues.

Another possibility is that both the degradation and formation of these two peptides are very slow and that changes of their relative concentrations in the muscles of the rat will only be noticeable after periods of dietary deficiency even longer than were used in the present work. No information is available as to the speed of synthesis or hydrolysis of the peptide linkage of anserine and carnosine *in vivo*, except for the results of Schenck, Simmonds, Cohn, Stevens & du Vigneaud (1943). These authors indicate that methylation at least is a relatively slow process. They fed methionine containing deuterium in the methyl group, and found anserine which was isolated from the muscles to have only a low deuterium content.

In this connexion it must be pointed out that our failure to detect any marked changes in the concentration of anserine in the muscles of the rat with various dietary deficiencies is in striking contrast to the findings of Hunter (1925), who investigated the factors affecting the concentration of carnosine in the muscles of the cat. This author observed that the carnosine content of striated muscles can be lowered by starvation and then raised again by a meat diet. It is possible that the level of carnosine in muscle is much more suceptible to dietary changes and this is important in the cat, where the concentration of carnosine, at least in some muscles is normally quite high. In the rat anserine which may be metabolically more inert and not be greatly affected by dietary restrictions, predominates and the β-alanine content may therefore be almost normal, although the carnosine is reduced. Such an explanation which assumes that the methylation or rather demethylation of the iminazole-N is the slowest step in the metabolism of anserine is supported by the fact that the carnosine figures found for histidine-deficient rats were significantly lower than those obtained on rats reared on normal diets.

SUMMARY

1. It has been shown that rats require the presence of histidine in the diet for the maintenance of body weight. The histidine requirements of other species have also been discussed, and it is suggested that the organism may be able to maintain a positive nitrogen balance for a limited period, although an amino-acid which cannot be synthesized by the body is absent from the diet.

2. The general effects of this dietary deficiency were found to consist of anaemia, hypoproteinaemia and loss of weight. The mildness of the anaemia is stressed, and it is suggested that the general result of a histidine deficiency is inhibition of protein synthesis similar in character but milder in degree than that found in most other deficiencies of essential amino-acids. It is concluded that the irreversible degradation of histidine proceeds at a slow rate.

3. The microbiological estimation of β-alanine has been used to estimate the combined carnosine and anserine contents of muscle extracts and of whole rats. It has been shown that at least 95% of the total β-alanine of the rat is present in protein-free extracts of muscles. Inhibition of the assay method by α-amino-acids has been considered and been found to affect the results under certain conditions.

4. Values for the carnosine and anserine contents of rat muscles were obtained which were similar, but on the whole lower than the figures found with other methods. Histidine deficiency was found to reduce the carnosine content quite appreciably, whilst the anserine values were either not affected at all, or only slightly. Possible explanations for these findings are discussed.

The authors wish to thank Dr P. Eggleton for a gift of anserine.

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Metabolic Processes in the Gastric Mucosa

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The metabolic properties of the digestive tract have received attention from time to time, notably those of certain parts of the intestines (Dickens & Weil-Malherbe, 1941); but there is little recent work on the respiration and glycolysis of the gastric tissues apart from Rosenthal & Lasnitzki's (1928) measurements on the mucous membrane. Extensive studies on the localization of peptic and hydrolytic enzymes were carried out by Linderstrøm-Lang and his associates (relevant papers quoted by Rask-Nielsen, 1944); recently the presence of carbonic anhydrase was demonstrated in the gastric mucous membrane by Davenport (1939) and the enzyme was extracted from this material and purified by Keilin & Mann (1940).

In the present study, rather than to pursue the investigation of enzymes concerned with digestive processes in the stomach, an attempt was made to gather information about the respiration and glycolysis of the mucosa and the other gastric components so as to establish the metabolic requirements of the healthy normal mucosa and to gain insight into conditions which may lead to pathological changes such as erosions and ulcerations.

Certain aspects of the metabolism of the gastric mucosa were examined in 1944 in the course of research concerned with the mechanism of systemic poisoning with mustard gas (Lutwak-Mann, 1946); these studies were undertaken on behalf of the Ministry of Supply and the results were summarized by Dixon & Needham (1946) but full details will be published later. A part of the present work was communicated to the Biochemical Society (Lutwak-Mann & Barrett, 1946).

EXPERIMENTAL

Material. Rats were chosen as experimental animals. The anatomy of the rat stomach, an account of which may be found in a paper by Berg (1942), differs somewhat from the human, but it is probable that findings based on the behaviour of the rat stomach will have general application.

Usually animals weighing 100–150 g., but also younger and older rats, were examined. The rats received a mixed stock diet except in some experiments on special diets mentioned later. They were killed by decapitation, bled, and the whole stomach removed and placed in saline. The methods of preparing the tissue for an experiment varied and as the results depend to some extent upon the technique employed, the details are given below.

Methods. Anaerobic acid production and O_2-consumption were measured in Barcroft manometers with side bulbs and gas outlets. Ringer-phosphate (Krebs, 1933) was used for aerobic experiments; phosphate was replaced by bicarbonate for the assay of acid under anaerobic conditions in an atmosphere of 95% N_2+5% CO_2. Unless otherwise stated, the volume of the fluid in the manometer cups was 2 ml. and the dry weight of the tissue between 8 and 10 mg. The concentration of glucose or other metabolites added to saline was 0·2%. Volatile substances, e.g. alcohols, were placed in equal amounts in both sides of the manometric vessels.

Lactic acid was determined by the method of Friedemann, Cotonio & Shaffer (1927), phosphate by the method of Fiske & Subbarow (1925), N was determined by means of the Kjeldahl modification of Parnas & Wagner (1921), the content of adenosinetriphosphate (ATP) by the method of Parnas & Lutwak-Mann (1935), polypeptide-N following the procedure of Burstein (1937), the content of glutathione and ascorbic acid according to Hopkins & Morgan (1936).

RESULTS

Anaerobic glycolysis

Gastric mucosa detached from the rest of the stomach wall

As the gastric mucous membrane does not lend itself to the application of the ordinary slice technique for manometric experiments, the following procedure was employed in the earlier stages of this work.

The stomach was opened along the greater curvature and very thoroughly rinsed in several changes of saline. It was

spread on the convex surface of a large watch-glass, the loose mucus was gently wiped off and the mucosa itself separated from the rest of the stomach wall with a small scalpel. Some laceration was unavoidable, but it was noted that the removal of the mucous membrane was much easier in stomachs filled to capacity with food digest than in the contracted stomachs of the starved rats. The material from one or, if necessary, from two or more stomachs was pooled and divided into roughly equal parts (equivalent to 10–15 mg. dry weight) and at once immersed in Ringer solution. To ensure the even distribution of the tissue, which tends to form clumps, a thin glass rod was used to unfold the pieces of mucous membrane in the manometer cups.

In the course of anaerobic incubation the mucosa gradually disintegrated and could not at the end of longer experiments be collected quantitatively. Therefore the contents of the manometer cups were emptied into centrifuge tubes and the deposit obtained after spinning was dried for 2 hr. at 110°. The values obtained in this way served as basis for the calculation of the earlier manometric results. However, it was later observed that even after as little as 20–30 min. anaerobiosis the saline in the manometer cup became increasingly opalescent and viscous, pointing to diffusion of protein from the tissue. To assess roughly the amount of soluble protein in the Ringer solution at the end of each experiment, after the removal of the tissue by spinning, dilute acetic acid was added dropwise to the supernatant fluid till no further precipitate was formed. The weight of this precipitate was determined after centrifugation and drying at 110°. The loss of protein from the gastric mucous membrane into the surrounding fluid during 1–2 hr. anaerobiosis was variable and amounted to between 20 and 50% of the whole dry weight. It also became clear that the larger losses occurred mostly in glucose-free samples, while in the presence of glucose as a rule less than 20% of the dry weight was attributable to the proteins which diffused into saline. The determination of 'initial' wet weight of tissue, apart from being time-consuming when speed was essential, was not practicable, owing to varying amounts of saline and loose mucus which adhered to the tissue after the rinsing in saline to remove food particles.

The graphs representing the progress of anaerobic acid production by the detached mucosa (Fig. 1) show that 20–30 min. from the start, the glycolysis, even in the presence of glucose, began to fall off. This could not be counteracted effectively by the addition of cozymase or a mixture of cozymase plus nicotinic amide; although there was some initial improvement in the rate of acid production, the effect of the coenzymes soon wore off. The addition to the Ringer solution of 0·2% glucose had some beneficial influence upon the maintenance of the tissue structure as judged by the smaller amounts of protein found in the saline, but the rate of acid production was either only slightly raised or not affected at all.

Even poorer glycolysis was obtained when, after separation from the stomach wall, the mucosa was ground into a uniform suspension (Fig. 1); the attempt to boost the low acid production by means of coenzymes was not successful, probably owing to destruction of these substances by enzymes liberated from the homogenized tissue.

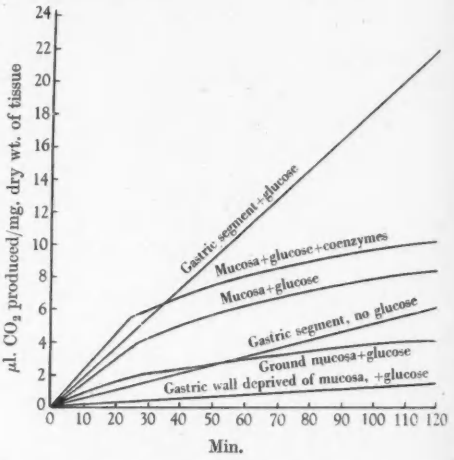

Fig. 1. Anaerobic glycolysis of gastric mucosa. Tissue incubated in Ringer-bicarbonate at 38°, in the form of segments of whole gastric wall, as mucosa detached from the rest of the stomach wall, in form of mucosa ground into uniform suspension, and stomach wall deprived mechanically of its mucous lining.

The anaerobic glycolysis of the separated gastric mucosa was not much improved when serum-bicarbonate was used instead of Ringer-bicarbonate. In view of the possible interference by serum with the determination of substances diffusing into the incubation fluid from the tissue, and the variable composition of serum, it was decided to employ Ringer solution rather than serum.

Segments of the whole stomach wall

The relatively rapid breakdown of the detached mucosa in anaerobic conditions, reflected in the progressive fall of glycolysis, made it desirable to find a more stable preparation. To this end, the gastric tissue was treated as follows.

The fore-stomach part was cut away and rejected, food particles were removed from the mucosa-lined portion of the stomach, and with very sharp scissors narrow (1·0–1·2 mm.) transverse annular segments of the entire wall were cut and washed in saline, till free from adhering food digest. During this, spontaneous eversion of these rings of tissue took place so that the outer circumference was that covered with the mucous lining. Usually sections were made in the region of the cardiac glands but in some experiments similar rings of gastric tissue were cut nearer the pyloric end of the stomach. With some practice it was possible to obtain segments the dry weight of which did not exceed 10 mg.

When gastric tissue prepared in this manner was used for manometric measurements of anaerobic glycolysis in Ringer-bicarbonate containing glucose, there was perfectly steady acid production (Fig. 1) for at least 2–4 hr. At the end of a 2 hr. period the saline surrounding the gastric segments remained almost water-clear; no debris of broken-up tissue was encountered and upon addition of acetic or trichloroacetic acid there was only slight precipitation of protein. Segments made from the same stomach showed good agreement but there were individual variations in the extent of anaerobic glycolysis when the gastric tissue of several batches of rats was examined.

In the absence of glucose, however, there was a marked difference in the behaviour of the tissue. The acid production was poor (Fig. 1) when anoxia had proceeded for 1–2 hr. and there was progressive disintegration of the tissue leading to a loosening of the surface epithelium which could be seen floating like a thin film still partly adhering to the deeper layers of the mucous membrane. At the same time the Ringer solution became opalescent and very faintly yellow. It was observed that gastric mucosa incubated in glucose retained its original reddish hue even after some hours of anaerobic incubation whereas the aglucotic material was almost colourless, confirming the loss of pigment to the solution.

When the glycolysis of sections of stomach wall from which the mucous lining had been mechanically removed was measured, very low values were obtained even in the presence of glucose (Fig. 1). In view of this it was felt justifiable to regard the values obtained with the gastric segments of the entire wall as representing chiefly the metabolism of the mucosa itself. It will be seen later that, deprived of the mucosa, the stomach wall was also incapable of utilizing O_2.

The optimum range of pH for the anaerobic glycolysis was rather narrow; best results were obtained between pH 7·4 and 7·8, but below pH 7·0 there was a considerable fall and hardly any glycolysis took place in unbuffered Ringer solution. The reaction proceeded in veronal buffer of pH 7·4–7·6, but less well than in bicarbonate or in phosphate buffer.

Lactic acid

The deproteinization with trichloroacetic acid of experimental samples which contained the separated mucosa invariably proved troublesome and no clear filtrates could be obtained. But there were no such difficulties with the gastric segments and these were used for the chemical determination of lactic acid. Table 1 shows the amount of lactic acid produced by gastric segments in the course of 2 hr. incubation in the presence of various substrates. With glucose or mannose the same quantities of lactic acid were formed, but in their absence the gastric mucosa was incapable of producing more than traces of lactic acid. Fructose, galactose, glycogen, also the Cori, Neuberg and Embden hexosephosphate esters, were poorly glycolyzed. The quantity of lactic acid as estimated chemically represented usually 70–85 % of the acid production measured manometrically. When segments were cut from the pyloric region of the stomach the discrepancy was somewhat bigger, the yield of lactic acid being as much as 40 % lower in some experiments than the amount of CO_2 produced. The possibility of another acid besides lactic acid, being produced anaerobically is not entirely excluded.

Table 1. *Anaerobic glycolysis in rat gastric mucosa in the presence of various substrates*

(Gastric segments from normal rats.
Incubation 2 hr.; 38°; Ringer-bicarbonate; 95 % N_2 + 5 % CO_2, 0·2 % substrate.)

	Acid formation/10 mg. dry weight of tissue	
Substrate	Lactic acid (mg.)	CO_2 (μl.)
Glucose	0·68	220
Mannose	0·70	220
Fructose	0·26	80
Galactose	0·23	70
Glycogen	0·30	78
Cori ester	0·38	90
Embden ester	0·30	85
Neuberg ester	0·29	92
No addition	0·10	46

Influence of certain external factors on the anaerobic glycolysis of the gastric mucosa

Several experiments were carried out in which groups of 12 rats each were kept for some weeks on inadequate diets and on diets deficient in vitamins. Some groups received a full synthetic diet to which sulphanilamide or sulphasuxidine were added daily. In others the effect was studied of ether anaesthesia when relatively large amounts of ether were inhaled and presumably a proportion of ether entered the stomach. In addition, various age groups and differences between sexes were investigated.

No clear-cut difference was noticeable as regards the sexes, but the glycolysis of the gastric mucosa was undoubtedly higher when young animals were used. The results of the experiments with various diets are summarized in Table 2. When the rats were kept on a protein-free, high carbohydrate diet, they lost weight but the glycolysis was not greatly affected, even after several weeks. Rats fared very badly on a high-fat diet; the appearance of the gastric mucosa was pale with the folds almost smoothed out; the values for anaerobic glycolysis

7–10 days after the start of the fat diet were much less than the control values.

Rats maintained on a diet deficient in vitamin B_1 showed all the usual deficiency symptoms, but the gastric glycolysis remained largely unchanged until the late stage when the rats were moribund. After 2 months of a vitamin A-deficient diet the glycolysis of the gastric mucosa appeared normal.

The daily addition, for several weeks, of 1 % sulphanilamide caused some slight wavering in the weight curves but generally the rats tolerated the drug well and the glycolysis remained unchanged. The addition of 0·5 % sulphasuxidine produced diarrhoea after 2 weeks of feeding; in most rats the gastric glycolysis was normal but after continued treatment with the drug it fell in some animals. Brief ether anaesthesia, sufficient to induce sleep, but not lethal, had no effect.

Table 2. *Effect of various diets on the anaerobic glycolytic activity of rat gastric mucosa*

(Gastric segments from rats on various diets; 12 animals in each group.
Incubation 2 hr.; 38°; Ringer-bicarbonate; 0·2 % glucose, 95 % N_2 + 5 % CO_2.)

Diet	Duration (weeks)	Acid formation/10 mg. dry weight of tissue: Lactic acid (mg.)	Acid formation/10 mg. dry weight of tissue: CO_2 (μl.)
High carbohydrate	2	0·68	210
High carbohydrate	4	0·58	200
High fat	1	0·50	180
High fat	3	0·38	140
Full synthetic + 1 % sulphanilamide	1	0·72	210
Full synthetic + 1 % sulphanilamide	3	0·60	200
Full synthetic + 0·5 % sulphasuxidine	1	0·63	200
Full synthetic + 0·5 % sulphasuxidine	3	0·47	185
Vitamin B_1-deficient	1	0·74	240
Vitamin B_1-deficient	2	0·67	220
Vitamin B_1-deficient	3	0·40	160
Vitamin A-deficient	10	0·66	190

The effect of substances added in vitro

The anaerobic glycolysis of the gastric mucosa was inhibited by 0·002 M-iodoacetate and 0·01 M-NaF. In the presence of these inhibitors of glycolysis the tissue suffered gross visible damage in spite of the presence of glucose. But the degree of disintegration appeared less severe than in gastric segments incubated anaerobically in glucose-free Ringer solution.

Atropine sulphate in low concentrations, 0·002–0·01 M, did not alter the course of glycolysis; ten or twenty times this amount had a slight inhibitory effect. The injection of 0·5 mg. atropine/g. of body weight was well tolerated by the rats; their gastric glycolysis was examined at various intervals after the injections but the values obtained did not differ from controls. The addition *in vitro* of sulphanilamide (15 mg./2 ml. Ringer solution) had no effect. Dodecyl sulphate 0·08 % caused a marked change in the appearance of the gastric segments making them look shrunken and almost translucent; the surrounding saline was rather viscous and glycolysis was about half the normal value. The addition of 10–15 mg. of ethanol or methanol to 2 ml. glucose-containing Ringer-bicarbonate did not diminish the rate of glycolysis; amyl alcohol (1 ml. shaken with 9 ml. water; 0·2 ml. used per experiment) depressed the glycolysis perceptibly. When the acid production was measured in the presence of 15 mg. of ethyl or methyl acetate/2 ml. saline, with the usual amount of glucose, variable results were obtained; in some instances there was moderate inhibition, in others the acid production surpassed the controls by about 20 %.

The effect of aglucotic anaerobiosis upon the structural and enzymic make-up of the gastric mucosa

Gastric mucosa exposed to lack of O_2 coupled with the absence of a glycolyzable substrate such as glucose or mannose showed, as previously described, macroscopic evidence of serious damage, but it was desirable to locate more precisely the lesions thus caused. To this end a histological examination was undertaken by Dr A. M. Barrett the results of which are summarized in the Appendix. To explore the possibility of an accompanying biochemical trauma the following type of experiment was carried out.

Gastric segments from normal young animals were prepared and incubated in the usual manner in manometer cups in Ringer-bicarbonate, with and without glucose, for varying lengths of time during which the glycolysis was measured. Then they were quickly transferred to fresh, glucose-containing Ringer-bicarbonate and the further progress of glycolysis was again recorded manometrically as well as by measuring lactic acid. In such experiments the glucose-containing samples gave invariably almost water-clear saline, even after a total incubation period of 4 hr.; but in the absence of glucose tissue debris was noticeable 1–2 hr. after the beginning of the incubation in the sugar-free saline, which itself became increasingly opalescent. Contrary to earlier expectations, this behaviour, far from being alleviated or reversed, was intensified by the transfer of the aglucotic tissue to fresh glucose saline. In fact, the degree of destruction wrought by aglucotic anoxia was less during an unbroken period of 4 hr. without glucose, than when such tissue was placed in glucose at some point of the

anaerobic incubation. This was not merely judged from the gross appearance of the experimental samples but was confirmed by the estimation of N in the saline.

The formation of lactic acid continued at a steady rate throughout the entire experiment in glucose or mannose, a little more lactic acid being found after transfer of the tissue to fresh Ringer solution. But the aglucotic tissue, which itself formed only traces of lactic acid, when removed to fresh saline containing glucose, was no longer capable of transforming it into lactic acid. Complete loss of glycolytic activity in the segments of the gastric wall occurred usually after 1–2 hr. in N_2 without glucose; but the material from young rats appeared more resistant and after 1 hr. such gastric tissue still retained most of its glycolytic power. The same results were obtained if phosphate buffer was used instead of Ringer-bicarbonate, the mixture of $CO_2 + N_2$ being replaced by N_2.

Table 3. *Diffusion of lactic acid, phosphate, ammonia and nitrogen from gastric rings into saline in the course of anaerobic incubation*

(Gastric segments incubated A, with glucose, and B, without glucose for 2 hr. at 38°.
Both A and B segments transferred to fresh Ringer-bicarbonate with 0·2% glucose, for a second incubation of 2 hr. at 38°. Results as mg./100 g. dry weight of tissue.)

	First incubation 2 hr.		Second incubation 2 hr.		Total experimental period 4 hr.	
	A (mg.)	B (mg.)	A (mg.)	B (mg.)	A (mg.)	B (mg.)
Lactic acid	6·0	0·8	8·0	1·1	14·0	1·9
Inorganic-P	0·16	0·25	0·09	0·15	0·25	0·40
Ammonia-N	0·035	0·031	0·005	0·017	0·040	0·048
Nitrogen	1·6	2·2	2·0	3·7	3·6	5·9

The salines at the end of incubation were examined for their content of P, ammonia-N and N (see Table 3). The gastric tissue deprived of glucose for 2 hr. had lost its ability to produce lactic acid from glucose. The amount of N which leaked into the saline was magnified by the transfer from glucose-free to glucose-containing saline. The total amounts of inorganic-P and ammonia-N which diffused into the fluid were higher in the experiments in which the glycolytic activity of the gastric mucosa had been destroyed as a result of aglucotic anoxia.

The content of pyrophosphate-P (hydrolyzable in 7 min. by N-HCl at 100°) in the gastric tissue during its anaerobic incubation was also examined. In the presence of glucose or mannose, the value of this labile P fraction fell after 30 min. incubation to less than half the amount found in fresh tissue (see p. 26). It continued to diminish progressively, reaching a low but apparently constant level. In the absence of glycolyzable sugars only a mere trace of pyrophosphate was detectable after the 30 min. interval, when material damage was already perceptible; at later stages of aglucotic exposure no pyrophosphate was found. The addition of glucose or mannose in the course of sugar-free incubation, or transfer to fresh glucose-containing saline, failed to bring about the reappearance of this P fraction.

It was important to establish the amount of glucose (or mannose) required by the rat gastric mucosa under anaerobic conditions for preservation of its structure and ability to form lactic acid from carbohydrate. A search was also made for substances which could replace glucose or mannose. To offset the suspected loss into the saline of labile coenzymes, various substances known to be essential for glycolysis were added alone or in combinations. Fig. 2 shows that as little as 1 mg. glucose (roughly M/400 under the conditions of the experiment) was already protective, but far better results were achieved using five to ten times more; excess beyond this led to little further protection of glycolysis. Fructose, galactose, glycogen, or the hexosephosphoric esters of Cori, Neuberg and Embden, were unable to secure the maintenance of glycolytic activity or to check the disintegration of the tissue. The only substitute for glucose was mannose, which preserved both the structure and the glycolytic system under anaerobic conditions. Some beneficial effect upon the acid production was observed when a mixture of cozymase, ATP and ADP (0·1 mg. of each/2 ml. fluid) was added to saline in which the aglucotic sample was incubated. Nicotinic amide and guanosine were without effect. Among other substances, examined in similar experiments, lactate and pyruvate as well as *dl*-methionine exerted a small positive effect, but the addition of glucosamine-HCl, *N*-acetyl-glucosamine or K hyaluronate, did nothing to protect the structural integrity of the mucous membrane or its glycolytic activity.

In view of the inability of the rat gastric mucosa to convert fructose to lactic acid, the possibility was examined of an interference with the action of glucose. Various concentrations of fructose were added to glucose but did not interfere with its protective effect (Fig. 2).

The Ringer-bicarbonate, in which gastric segments were incubated anaerobically without glucose for 3–4 hr. till it became opalescent and tissue debris accumulated, was collected and replenished with glucose and then used as medium for a manometric experiment with fresh gastric segments. The fact that glycolysis was unimpeded proved that there had been no leakage of substances from the asphyxiated aglucotic tissue, capable of interfering with the glycolysis.

The ability of the mucosa to respire was examined after various intervals of anaerobic aglucotic incubation. After 60–80 min. the tissue was still fully capable of taking up O_2, but longer anaerobic exposure caused significant decrease in the O_2 consumption.

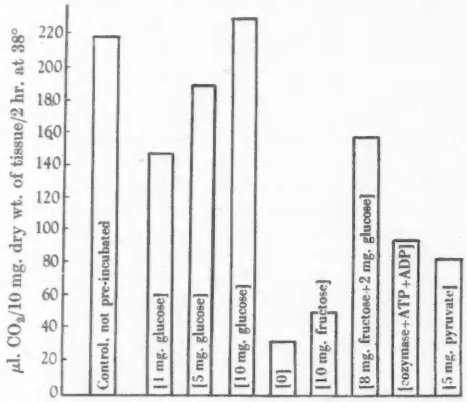

Fig. 2. Glycolytic activity in the gastric mucosa after a preceding period of anoxia. After 2 hr. anaerobic preincubation with various substances [indicated in square brackets] the gastric segments were transferred to fresh Ringer-bicarbonate containing 0·2% glucose and the anaerobic acid production measured for 2 hr. at 38°. (Average data from 4 experiments each.)

The possibility was considered that while enzymic systems dependent upon labile coenzymes might suffer irreparable damage from exposure to anaerobic conditions without utilizable substrate, others might fare much better, either because they were situated in less vulnerable layers of the gastric mucosa, or owing to greater inherent resistance. It was decided to explore the fate of carbonic anhydrase which is present in the parietal cells of the gastric mucous membrane (Davenport, 1939). The experiments were arranged in the following manner.

The stomachs of young rats were opened along the greater curvature, washed very thoroughly in saline, weighed and placed in manometer vessels in 5 ml. Ringer-bicarbonate or Ringer-phosphate, with or without glucose. They were kept for varying periods in strictly anaerobic conditions. At the end of the incubation the saline was quantitatively collected, the stomachs were rinsed with 5 ml. water which was added to the saline. The mucosa was then collected, weighed, ground with 3 ml. water and centrifuged; the residue was rejected, and the supernatant extract, as well as the previously collected saline+washings, were used for the assay of carbonic anhydrase by means of the colorimetric method of Philpot & Philpot (1936) as described by Keilin & Mann (1940). The main results were also confirmed with the manometric boat apparatus of Brinkmann, Margaria & Roughton (1933) following the procedure of Meldrum & Roughton (1933).

In the experiments recorded in Table 4 the wet weights of the whole stomachs did not differ appreciably (between 0·75 and 0·9 g. wet weight); the amounts of separated mucosa were equal (0·18 g. wet weight); and the same volumes of saline and water were used for each sample. It can be seen that, whereas after 2 hr. in anaerobic conditions carbonic anhydrase was detected in the incubation fluid both in the presence and absence of glucose, distinctly more enzyme was found in the glucose-free saline. When the incubation period was extended to 4 hr. there was several times more enzyme in the absence than in the presence of glucose. The enzyme in the aqueous extract from the mucosa itself was little affected by 2 hr. anaerobiosis, but after longer exposure there appeared to be some loss of activity in the mucosa deprived of glucose. In analogous experiments carried out aerobically, after 2 hr. incubation in O_2 with and without glucose, little more than traces of carbonic anhydrase activity were found in the saline. Somewhat more was detectable after longer incubation, but the dependence upon glucose was slight. The figures for the N content of the saline samples show that under anaerobic conditions much more N was lost into the fluid surrounding the tissue than in O_2, and how this loss of N was magnified by the lack of glucose (Table 4).

Table 4. *Behaviour of carbonic anhydrase in the gastric mucosa during anaerobic and aerobic incubation*

(Rat stomachs incubated in 5 ml. saline, with and without glucose, for 2 hr. and 4 hr. at 38°. After incubation, rinsed with 5 ml. water. Saline + rinsing water from each experiment combined, and used for enzyme assay as 'saline' with and without glucose. Mucosa ground in 3 ml. water, aqueous extract used for assay as 'mucosa' with and without glucose. The colorimetric test of Philpot & Philpot (1936) was used to determine the activity of carbonic anhydrase in these fluids.)

Material examined	Enzyme activity as vol. of fluid required to complete colorimetric test in 30 sec. Stomachs incubated 2 hr. (ml.)	Stomachs incubated 4 hr. (ml.)	N content in 'saline' after 4 hr. incubation (mg.)
1. Anaerobic incubation			
'Saline' with glucose	2·0	1·6	2·7
'Saline' without glucose	0·8	0·4	4·4
'Mucosa' with glucose	0·2	0·15	—
'Mucosa' without glucose	0·2	0·30	—
2. Aerobic incubation			
'Saline' with glucose	3·0	2·0	1·6
'Saline' without glucose	2·5	1·4	1·9
'Mucosa' with glucose	0·15	0·15	—
'Mucosa' without glucose	0·10	0·15	—

Oxygen uptake

Aerobically, the behaviour of the gastric tissue differed in several aspects from that exhibited in the absence of O_2. The gastric mucosa separated mechanically from the rest of the stomach wall was clearly capable of preserving its coherence without undue loss of protein into the surrounding saline, even in the course of fairly long aerobic incubation. Secondly, the ability to maintain satisfactorily the structure was largely independent of the addition of glucose, except during very long incubation periods when a slight beneficial effect was noticeable.

When, instead of the detached mucosa, circular sections or simply thin strips of the whole wall were incubated in saline in O_2, there were even fewer signs of tissue damage; the diffusion of N into saline was much less than in anaerobic experiments, and the effect of glucose negligible. Some of the earlier experiments were carried out in air, as it was felt that the gastric mucous membrane might be sensitive to pure O_2. However, it would appear from the histological findings that in air there may have been at least partial anaerobiosis which expressed itself as loss of surface epithelium (see Appendix), a phenomenon found to be characteristic of gastric mucosa suffering from lack of O_2. As there was no evidence of any special sensitivity of the mucosa to pure O_2, all later experiments were done in O_2, but the duration of the routine experiments was limited to 2 hr. to minimize the risk of microbial contamination. The O_2 uptake in air was found to be several times less than in pure O_2, both with segments of the whole gastric wall and with the detached mucosa which consumed only about $\frac{1}{3}$ to $\frac{1}{2}$ of the amount utilized in O_2.

Except in very young rats, the thickness of the stomach wall was greater than 1 mm.; none the less, when very thin (0·8–1·0 mm.) strips were prepared, their dry weight not exceeding 6–8 mg., satisfactory and reproducible manometric results were obtainable (Fig. 3), while at the same time the structure of the tissue as a whole was reasonably well preserved. Moreover, the respiration of the stomach wall from which the mucous lining had been removed represented not more than 10% of the values reached by the segments with their mucosa intact (Fig. 3). The detached mucosa (10–15 mg. dry weight) consumed less O_2 than segments of the entire wall and the reaction was not linear beyond 1 hr. (Fig. 3).

Substrate

Practically the same results were obtained whether or not glucose was added, both with detached mucosa and whole stomach wall. Occasionally, an enhancing influence of glucose upon the O_2 uptake was observed, particularly in starved rats, and in old animals in which the gastric mucosa showed generally lower values than in young rats (Fig. 3), but the respiration of the detached mucosa was sometimes actually depressed by the addition of glucose. The addition of fructose to both types of tissue preparation slightly lowered the O_2 consumption. The addition to Ringer-phosphate of succinate, glucosamine-HCl, *N*-acetylglucosamine, had no effect upon the respiration of the segments of gastric mucosa.

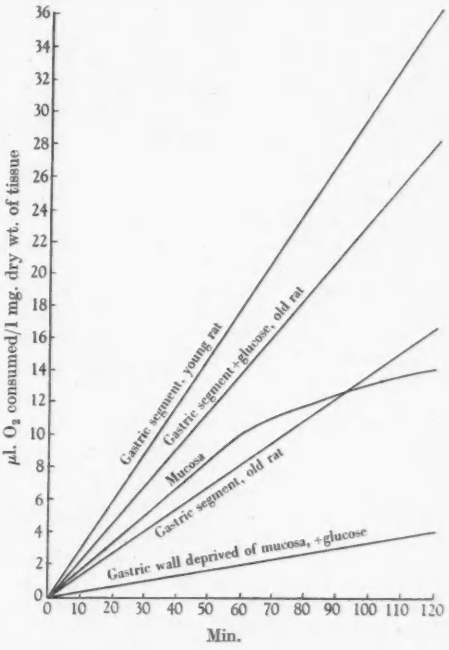

Fig. 3. O_2 uptake of gastric mucosa. Tissue incubated in Ringer-phosphate at 38°. Segments of entire stomach wall, mucosa separated from the wall, and stomach wall deprived of mucous membrane.

Unlike the anaerobic glycolysis, the O_2-uptake was not excessively sensitive to changes of pH, values obtained between pH 6·5 and 7·8 differed little. The effect of various diets, similar to those already described, upon the utilization of O_2 by the gastric mucosa was also examined. Generally, it was found that where the animals suffered badly from inadequate food composition, the O_2 consumption was less than in control rats; otherwise, there were no significant changes.

Aerobic glycolysis

Some lactic acid was produced by the gastric mucosa from added glucose during incubation in O_2. The amounts found at the end of a 1 or 2 hr. period represented about half or even less of the values formed in corresponding experiments in the absence of O_2. The aerobic glycolysis was slightly higher in Ringer-phosphate than in Ringer-bicarbonate. Without added glucose, the respiring mucosa did not produce any lactic acid.

The effect of inhibitors and other substances added in vitro

There were some differences in the response to inhibitors, as between the separated mucosa and the gastric segments. In presence of KCN (0·001 M) the respiration of the gastric mucosa was at a standstill (Fig. 4); weak NaF (0·0025–0·01 M) had little

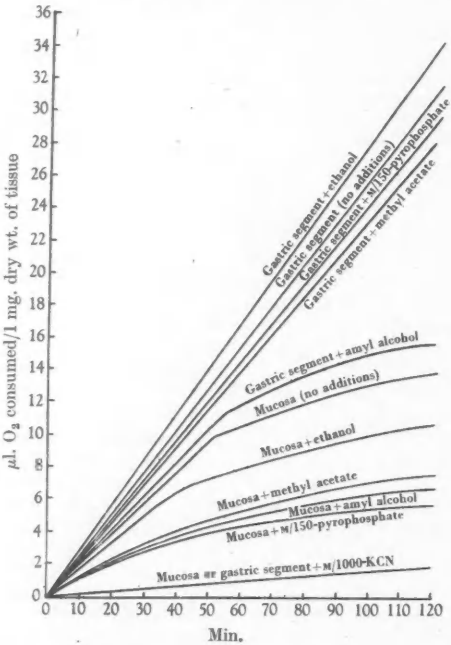

Fig. 4. Effect of various substances on O_2-uptake of gastric mucosa. Tissue incubated in Ringer-phosphate at 38°. Segments of whole stomach wall, or mucosa alone. Concentration of alcohols or esters, 15 mg./2 ml. saline.

affect on the respiration of the gastric strips but decreased that of the separated mucosa; NaF (0·0125 M) considerably inhibited O_2 uptake in both kinds of tissue preparation. Pyrophosphate (0·005–0·01 M) did not alter the respiration of gastric segments but caused considerable inhibition of separated respiring mucosa (Fig. 4). Substances which inhibited the O_2 uptake of the entire wall and the detached mucosa, were: 0·08% dodecyl sulphate; 0·005 M-Na_2S; 0·005 M-dimethyldithiocarbamate; malonate, 0·005–0·01 M, was without effect, but in higher concentration (0·02 M) inhibited the respiration. Atropine in the amounts mentioned above had no effect. Ethyl or methyl acetate slightly inhibited gastric strips but decreased the O_2 uptake of the detached mucosa. Ethanol and methanol had either no effect on the gastric segments or slightly enhanced their O_2 consumption, but lowered significantly the O_2 uptake of the mucous membrane alone; amyl alcohol strongly inhibited the respiration regardless of the kind of tissue preparation (Fig. 4).

ASSAY OF THE CONTENT OF CERTAIN SUBSTANCES IN THE RAT GASTRIC MUCOSA

The content in the rat gastric mucosa of the following substances was estimated: adenosinetriphosphate (ATP), acid-soluble P fractions, polypeptide-N, glutathione, ascorbic acid, glycogen. In all experiments the rumen portion was rejected and the whole mucosa-covered part of the stomach was used without separating the mucous membrane from the rest of the wall. The material from 6 to 8 rats was used for each determination.

For both freshly fed and starved rats, 1·6 mg. ATP amino-N/100 g. wet weight of stomach was found. The values for acid-extractable P content/100 g. wet weight were: 50–60 mg. total acid-soluble P, of which 20–25 mg. was directly determinable, 6–8 mg. belonged to pyrophosphate-P, 5 mg. to esters hydrolyzable in 1 hr. and the rest was made up of difficulty acid-hydrolyzable P esters.

The total non-protein-N content of the rat gastric mucosa was 116 mg. and the polypeptide-N was 20 mg./100 g. wet weight of tissue.

The content of ascorbic acid and glutathione (GSH) was estimated in normal animals and also in rats maintained for 3 weeks on diets containing 1% sulphanilamide or 0·5% sulphasuxidine. The normal values were 0·65 mg. GSH and 0·13 mg. ascorbic acid/g. wet weight. In rats on diets containing sulphonamide drugs, the values for GSH were less than in controls, 0·42 and 0·51 mg., in the sulphanilamide- and sulphasuxidine-fed animals, respectively; but the amounts of ascorbic acid appeared unaltered.

In a few experiments the Pflüger procedure for the determination of glycogen was applied to rat gastric mucosa, but no precipitate was obtained after the addition of 1–2 vol. of ethanol to the

KOH hydrolyzate. When 10 vol. of ethanol were added the precipitate which formed was not glycogen.

DISCUSSION

The biochemical study of the metabolic properties of the digestive tract necessitated the adoption of an approach varying from that generally accepted as suited to the investigation of such organs, as for instance the liver. In its make-up the digestive tract lacks the uniformity of structure characteristic of liver tissue (in which one type of cell largely predominates) and is built from different interpenetrating morphological elements; attempts to separate them artificially for experimental purposes tend to cause a fall in enzymic processes of the tissue such as the respiration and glycolysis. The mucous membrane under physiological conditions comes into contact with metabolites and other substances not only through the blood-stream like all other tissues, but also from the surface lining of the lumen. In this respect, the gastric differs from the intestinal mucosa in that it comes into contact with largely undigested matter. It would not be surprising if the mucous membrane were to react differently according to whether substances reached its epithelium-lined surface facing the stomach cavity or entered the cells *via* the blood capillaries. Normally secretion of gastric mucin by the epithelial cells protects the exposed surface against noxious agents but when this function of mucin production is interrupted owing to material damage to the epithelium, the deeper layers of the stomach wall may easily suffer.

The great frailty of the gastric mucosa after separation from the rest of the stomach wall in absence of O_2, even when supplied with glucose, makes it unsuited for anaerobic experiments. The use of segments of the entire gastric wall meant that some precision in manometric technique had to be sacrificed, as their thickness mostly exceeded that considered optimal for manometric assays with tissues such as liver, kidney and brain. On the other hand, even when O_2 was excluded, they maintained for a time at least a reasonable degree of tissue integrity, reflected in the steady rate of the metabolic processes investigated. With the detached mucosa it was impossible to determine satisfactorily whether the addition of glucose increased the anaerobic acid production; but with the sections of the entire wall the results were clear-cut and gave unequivocal proof of increase by glucose (cf. Fig. 1). Fortunately, the stomach wall mechanically deprived of its mucous lining was almost without glycolytic and respiratory activity.

This investigation has shown the utter dependence of the normal gastric mucosa, under anaerobic conditions upon the presence of a glycolyzable substrate, both for the preservation of important enzymic systems (e.g. glycolysis) and for the maintenance of tissue integrity. The first tissue element to succumb in absence of O_2 and glucose appears to be the epithelium lining the surface of the mucosa, but deeper elements were also somewhat affected. The concentration of glucose which was found to be protective lay well within the physiological range, though in the experiments *in vitro* somewhat higher concentrations were required to achieve full protection for the preservation of the tissue and the glycolytic activity. The only substance which could replace glucose was mannose; other sugars, which are not glycolyzed by the rat gastric mucosa, did not protect.

The phenomenon of anaerobic dependence of tissues upon a glycolyzable carbohydrate is undoubtedly a general one and not limited to the gastric mucous membrane. Segments of caecum were also observed to be disintegrated and to lose their glycolytic activity as a consequence of exposure to aglucotic anaerobic conditions. Dickens & Greville (1933) similarly found that in brain slices even very brief anaerobic periods of substrate deprivation resulted in loss of glycolytic power and Macfarlane & Weil-Malherbe (1941) established the disappearance under such conditions of the pyrophosphate-P in brain tissue.

On the basis of the older work as well as the experiments described here, there is every reason to believe that the failure of the labile adenosinepolyphosphates to be resynthesized when the glycolytic cycle is interrupted, is an important factor explaining the loss of glucolytic activity by the anoxic tissue. But it still remains for future study, biochemical and cytological, to determine precisely the point of juncture between the chemical reactions involving the carbohydrate metabolism and the structural damage which seems to affect cell proteins and probably other complex components of the tissue, as soon as the supply of essential glycolytic coenzymes is exhausted. The altered and rapidly deteriorating cell-permeability of the gastric mucosa under anaerobic conditions was best indicated by the diffusion into the surrounding saline of an enzymic protein, carbonic anhydrase, the presence of which could sometimes be detected in the incubation fluid before the glycolysis of the mucosa was wholly destroyed. Another instance of the close relationship between structural integrity and glycolysis was provided by experiments with iodoacetate and NaF, in the presence of which tissue damage was clearly visible.

It is too early to speculate upon the possibility of erosions or even ulcerations being a direct outcome of such changes as were observed in the gastric mucosa when deprived of substrate anaerobically. But it is conceivable that owing, e.g., to temporary

breakdowns in blood circulation, especially in poorly vascularized areas, parts of the mucous surface might suffer losses in the continuity of the epithelial lining whereby gastric mucin would no longer be produced and chemical agents could penetrate into deeper layers.

In contrast to its behaviour under anaerobic conditions, the gastric mucosa in O_2 was found to be quite independent of glucose. The preformed substrate utilized aerobically by the mucosa, has not yet been identified. The sensitivity of the O_2 uptake to KCN makes it probable that the cytochrome system is involved in the respiration of the mucous membrane.

When as the result of a faulty diet the condition of the rats was poor, the metabolism of the gastric tissue was usually also lowered. A more detailed examination of very much larger numbers of animals and other species might perhaps reveal an association of metabolic changes with specific nutritional disorders.

SUMMARY

1. The anaerobic glycolysis and O_2 uptake of the gastric mucosa of the rat were studied. The experimental material was used either in the form of mucous membrane detached from the rest of the stomach wall or as segments of the entire wall. The stomach wall itself, freed from its mucous lining, had little glycolytic or respiratory activity.

2. The anaerobic glycolysis was best studied using segments of the whole gastric wall, because anaerobically the separated mucosa disintegrated rapidly. Glucose and mannose were readily converted into lactic acid by the gastric segments, but fructose, galactose, glycogen and several hexose-phosphates, were poorly glycolyzed. The glycolysis was inhibited by low concentrations of iodoacetate or NaF.

3. In absence of O_2 the maintenance of both tissue integrity and glycolytic activity in the gastric mucosa depended upon the presence of a glycolyzable substrate such as glucose or mannose, of which concentrations as low as 0·0025-M sufficed to prevent the structural damage and preserved the glycolytic system. Various substances examined were ineffective as substitutes for these two sugars.

4. Enzyme systems not dependent upon labile coenzymes were less susceptible to destruction under anaerobic aglucotic conditions. It was found that the respiration was little affected by preceding anaerobiosis; the gastric carbonic anhydrase was also resistant.

5. Aerobically, without glucose, the gastric mucosa maintained its enzymic activity and structure; the addition of glucose had no significant effect upon the rate of O_2 consumption. Low concentrations of KCN completely inhibited the respiration of the gastric mucosa. The rate of the aerobic glycolysis was not more than about half of that due to the lactic acid formed from glucose anaerobically.

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* Information available on application to Ministry of Supply, London.

Appendix. Histological Observations

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Some of the gastric ring sections used in the metabolic experiments were afterwards fixed in Heidenheim's 'Susa' and sent to me in 80% alcohol for histological examination. They were embedded in paraffin, and sections were stained by Ehrlich's haematoxylin and eosin.

Anaerobic experiments with and without glucose (tissue incubated 2–4 hr.). Three specimens from anaerobic experiments without glucose were examined. In two of them the greater part and, in the third, a smaller part, of the epithelium which should line the inner surface of the stomach and the gastric pits were missing. On the other hand, this surface epithelium was intact in all three specimens from comparable experiments in the presence of glucose. Other changes, e.g. separation of the zymogenic cells and focal necrosis in the mucosa, were present in most specimens and appeared unrelated to the presence or absence of glucose.

Aerobic experiments in air and oxygen (2–3 hr. incubation periods). Four specimens from experiments carried out in oxygen were compared with four specimens from comparable experiments in air. Yeast-like organisms were seen on the mucosal surface of all four 'oxygen' specimens, but not in any of the 'air' specimens. Swelling and rarefaction of the connective tissue immediately beneath the surface epithelium, an abnormality not seen in any anaerobic specimens, was present in three of the 'oxygen' specimens but only in one of the 'air' specimens. On the other hand, the surface epithelium was partially absent in two of the 'air' specimens but not in any of the 'oxygen' specimens. The significance of these differences is doubtful and no others were observed. They are commented upon on p. 25.

Two of the 'oxygen' specimens and two of the 'air' specimens were from experiments in which glucose was present; the other two of each from experiments in which glucose was absent. Swollen empty-looking muscle fibres were present in the muscle coat of all the 'glucose-present' specimens but not in any of the others. No other effect of the presence or absence of glucose in aerobic experiments was recognized.

The specimens from experiments in air or oxygen appeared better preserved on the whole than those from anaerobic experiments. In particular, pyknosis of the nuclei of parietal cells and focal necroses were less common in the 'aerobic' specimens.

A Modified Method for the Viscosimetric Assay of Hyaluronidase

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(*Received* 26 *July* 1946)

The method of McClean & Hale (1941) for the viscosimetric assay of hyaluronidase has been in extensive use and is claimed to be accurate to within ±10%; in the hands of the present authors, however, it has not proved entirely satisfactory. We have, therefore, been prompted to investigate some of the fundamental principles upon which the method is based and, as a result, have succeeded in devising a simpler and apparently more accurate procedure. The method of McClean & Hale is based on the work of Madinaveitia & Quibell (1940) who calculated the 'half viscosity time' or 'half-life time of the substrate', which they found to be inversely proportional to the concentration of the enzyme and independent of the concentration of the substrate, both of which conclusions were supported by McClean & Hale and made the basis for their viscosimetric technique. On the other hand, Robertson, Ropes & Bauer (1940) found that the time taken for a fixed amount of enzyme to break down increasing amounts of substrate was proportional to the concentration of the latter. In view of this apparent discrepancy, and since the time taken to reach the half viscosity level of the substrate did not seem to give a satisfactory index of the potency of the enzyme, it was decided to investigate (1) the relationship of the flow-time in Ostwald viscosimeters to the concentration of a simple viscous solution (dextrose); (2) the same relationship for buffered hyaluronate solutions of varying concentration; (3) the curve of flow-times for hyaluronate solutions mixed with hyaluronidase; and (4) the

curve of flow-times of a standard hyaluronate solution incubated for a constant time with varying concentrations of hyaluronidase.

EXPERIMENTAL

Materials and methods

The substrate consisted of a solution of potassium hyaluronate (Glaxo Laboratories), four volumes of which were mixed with one volume of McIlvaine's (1921) citric acid-phosphate buffer M/60, pH 7·0, containing NaCl; (Na_2HPO_4 [anhydrous] 12·75 g.; citric acid 2·02 g.; NaCl 3·50 g.; water to 1 l.). The hyaluronidase consisted of freeze-dried rabbit- and bull-semen filtrates, and all dilutions were made with 0·5% gum arabic solution, since a loss of potency on standing has been observed when distilled water alone is used as the diluent. The Ostwald viscosimeters were of about 1·5 ml. capacity, and all measurements were made in a water bath at 34°. The quantities of buffered substrate (2·5 ml.) and enzyme solution (0·5 ml.) were the same as those employed by McClean & Hale (1941).

The relationship of flow-time to the concentration of viscous solutions

The curves of flow-time plotted against concentration of dextrose and buffered hyaluronate solutions are shown in Fig. 1. (In Figs. 1 and 2 each

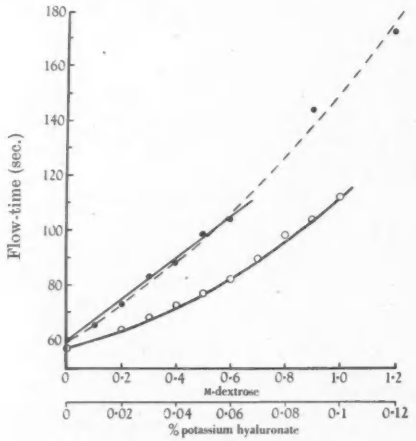

Fig. 1. Relationship between concentration of dextrose ○—○ and buffered hyaluronate ●—● solutions and flow-time in an Ostwald viscosimeter.

point represents one determination on the same viscosimeter.) It will be seen that they resemble one another in so far as flow-time is not directly proportional to concentration, increasing at a progressively greater rate as the concentration is increased, but they differ markedly in that, over the range of flow-times 60–110 sec. the curve for hyaluronate is practically a straight line, while that for dextrose is not. Within the above-mentioned range, therefore, it is permissible to regard the flow-time of a hyaluronate solution as being directly proportional to its concentration, and for this reason the concentration of hyaluronate used for viscosimetric assays has been adjusted so as to fall within this range.

The curve of flow-times of hyaluronate solutions mixed with hyaluronidase

It became apparent, as an important practical point, that the method of mixing the hyaluronidase solution with the substrate had a marked effect on the subsequent rate of fall of the flow-time of the mixture. Because of the viscosity of the substrate, and of the small volumes of fluid employed, diffusion is minimal and it is therefore imperative that mixing be thorough from the outset. Accordingly, a uniform method of adding the enzyme to the substrate (both solutions being at 34°) was adopted, namely: vigorously squirting the former into the latter from a 1 ml. all-glass syringe through a No. 26 needle. Scrupulous attention to this detail, as well as to ensuring the viscosimeter's being at 34°, was found to be essential for obtaining reproducible results. (The viscosimeter should have stood for at least 10 min. in the water-bath before adding the enzyme-substrate mixture.)

The effect of variation of substrate concentration on the curve of flow-times is seen in Fig. 2, where the 'relative viscosity' (McClean & Hale, 1941) is plotted against time after mixture of enzyme and substrate, the concentration of enzyme remaining constant. Contrary to the findings of Madinaveitia & Quibell, and of McClean & Hale, it is seen that the time to the half viscosity is not independent of the concentration of the substrate, but becomes progressively less as the latter is decreased. Although the discrepancy is not great with more concentrated substrate solutions, i.e. containing about 0·08% hyaluronate or more (such as the above authors have, for the most part, used), it is very prominent with more dilute substrates and is certainly appreciable within relatively narrow limits if the range of concentrations which we advocate (wherein flow-time is proportional to concentration) is used. It is clear that, although with more concentrated substrates the amount of substrate attacked per unit time by a given enzyme preparation may be roughly proportional to the original substrate concentration, with more dilute substrates this relationship fails to hold good.

The curve of flow-times of a standard hyaluronate solution incubated for a constant time with varying concentrations of hyaluronidase

In view of the above theoretical objection to the use of the time to the half-viscosity as a measure of the potency of hyaluronidase, as well as the practical one of the tediousness of its determination, it was decided to investigate the possibility of employing the flow-time of the enzyme-substrate mixture after incubation for a fixed period as the index of hyaluronidase concentration. It can be seen from the curve of flow-times (Fig. 2) that the rapidity of fall decreases progressively, the curve having flattened considerably by 20 min. This time, therefore, was considered to be a suitable and convenient one for practical use.

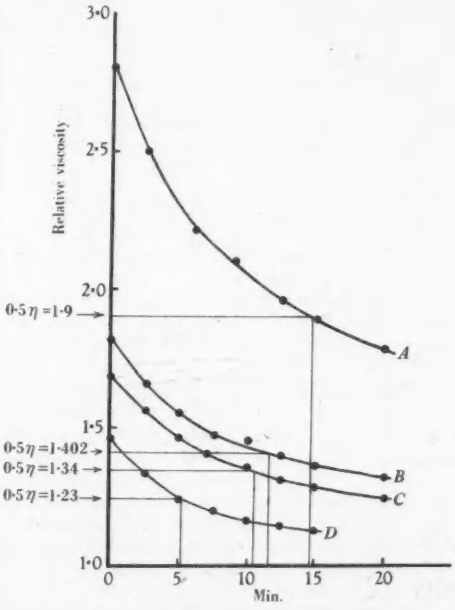

Fig. 2. Curves of flow-times of mixtures of a fixed concentration of hyaluronidase with varying concentrations of buffered hyaluronate, showing variation of 'time to the half-viscosity' with concentration of substrate. The concentrations of hyaluronate are *A*, 0·103 %; *B*, 0·066 %; *C*, 0·062 %; and *D*, 0·053 %.

Since the flow-times, not only of water, but also of the substrate varied somewhat in different viscosimeters, it seemed desirable to devise, if possible, a correction factor which would eliminate these differences and so enable one to make comparable readings on different tubes without always having to arrange tests so that differences between tubes could be eliminated statistically. The 'relative viscosity' of McClean & Hale (1941) is not entirely satisfactory in this respect, and the following considerations led to the adoption of the index which is advocated:

If f_s be the flow-time of the substrate + 0·5 ml. gum arabic solution in place of enzyme, f_o be the flow-time of the solvents without hyaluronate, and f_e be the flow-time of the enzyme-substrate mixture after incubation for 20 min., then $f_s - f_o$ represents the viscosity due to the substrate, while $f_s - f_e$ represents the amount of substrate rendered non-viscous by the action of the enzyme, and, within the limits described above, is directly proportional to the amount of substrate so attacked. It is reasonable to assume that the ratio $1000\left(\frac{f_s - f_e}{f_s - f_o}\right) = v$ (the constant 1000 avoids decimal points) will be constant for a given substrate-enzyme mixture in spite of variations (within limits) in the actual values of f_s and f_o, that is, in spite of slight tube-differences. This ratio, then, has been used as the flow-time index, and it will be shown by statistical analysis of test assays that the above assumption is true within the limits of experimental error.

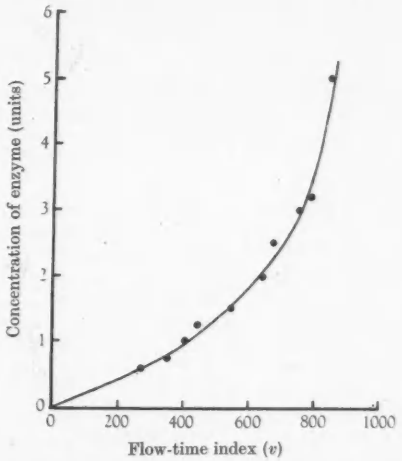

Fig. 3. Relationship between flow-time index (v) and relative concentration of enzyme for mixtures of hyaluronidase and buffered hyaluronate (the latter remaining constant) incubated for 20 min. at 34°.

Fig. 3 shows the relationship between concentration of enzyme and the flow-time index (v) for a standard hyaluronate solution incubated for 20 min. Although there is an asymptote to the curve at $v = 1000$, it is sufficiently close to the exponential form over the range $v = o - 800$ to warrant the

working assumption that the flow-time index is proportional to the logarithm of the enzyme concentration over this range (Fig. 4). The justification for this assumption will be shown later. The advantage of this relationship lies in the fact that it enables one to assay samples of enzyme in terms of a standard preparation as described by Bliss & Marks (1939), and for this purpose a laboratory standard

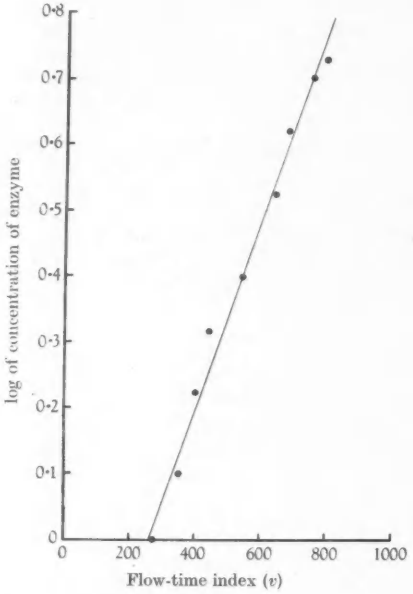

Fig. 4. Relationship between flow-time index (v) and logarithm of concentration of enzyme for the same conditions as in Fig. 3.

preparation has been set up. This consists of diluted bull-semen filtrate which has been ampouled and freeze-dried, each ampoule containing a constant quantity (about 0·1 ml. of the original semen filtrate). It is proposed to regard the contents of each ampoule as 10 units. The freeze-dried material has been found to weigh 8·5 mg. per ampoule and comparison with a Glaxo Laboratories enzyme preparation as used by McClean (1 mg./ml. ≡ 100 M.P.C. units; McClean, 1943) shows that 1 mg. of the latter is equivalent to 0·475 mg. of our freeze-dried material or 0·56 unit.

In practice, the method of assay consists in incubating the appropriate quantities of buffered hyaluronate with enzyme in two dilutions (one usually half that of the other) and determining the flow-times at the end of 20 min. This is done for each dilution in three tubes, and the process is repeated with two similar dilutions of standard preparation, so that in all, twelve readings are obtained. One ampoule of the standard made up to 40 ml. is a convenient higher dilution. From these readings one can calculate the relative potencies and estimate any departure from parallelism of the slopes of the dose-effect curves after the method of Bliss & Marks, while from the analysis of variance it is possible to estimate the standard error and the significance, if any, of differences between tubes.

Test and statistical analysis of the method

Following the above considerations, a test was set up in which four different concentrations of a preparation of rabbit semen filtrate were compared in three different viscosimeters. The test was completed in one day, and the order of testing concentrations in different tubes was at random. The results are shown in Table 1. The balance of the

Table 1. *Results of a test of hyaluronidase at four concentrations*

Concentration of hyaluronidase (dilution of standard)	Viscosimeter A		Viscosimeter B		Viscosimeter C	
	Flow-time	v	Flow-time	v	Flow-time	v
1/300	86	316	88	342	95	259
	86·5	303	87	366	92·5	318
	85	342	90	293	93·5	294
1/200	82	420	86·5	378	86	471
	81	447	82	488	87	447
	79	500	82·5	475	86	471
1/100	75·5	593	76	635	79·5	625
	76	580	76·5	622	79	635
	74·5	620	76	635	78·5	647
1/75	70·5	725	72·5	720	77·5	670
	71·5	700	74	685	76·5	695
	71	710	75	660	76	705

test is such that each concentration was tested three times in each viscosimeter, so that variation due to differences between instruments can be segregated in the analysis of variance, set out in Tables 2 and 3.

These two analyses of variance show that the highly significant differences between the flow-times of different viscosimeters were completely eliminated in this particular test by conversion to the index, v, and that no significant interaction of concentration with instruments occurred. A most satisfactory degree of significance of the slope of the line relating concentration to flow-time was obtained, the slope for the data of Table 3 being 620 and its standard error 6·6 (using the relative indices, 1, 1·5, 3 and 4, for the four concentrations).

Tests of goodness of fit show that the points do not deviate significantly from a straight line for the data of either Table 2 or 3, but that the fit for Table 3 is the better ($F = 2·81$). If the first and

Table 2. *Analysis of variance for a test of hyaluronidase at four concentrations, using actual flow-times*

Source of variation	Degrees of freedom	Mean square	*F*
Concentrations	3	431·1	312*
Viscosimeters	2	99·0	72*
1st order interaction	6	2·76	2·0
Error	24	1·38	—

* $P > 0{\cdot}99$.

Table 3. *Analysis of variance for a test of hyaluronidase at four concentrations, using the index v*

Source of variation	Degrees of freedom	Mean square	*F*
Concentrations	3	263327	293*
Viscosimeters	2	84·1	0·10
1st order interaction	6	1125	1·3
Error	24	842	—

* $P > 0{\cdot}99$.

third concentrations are assayed against the second and fourth, the logarithm of the relative potency and its standard error are:

(*a*) from actual flow-times, $0{\cdot}0221 \pm 0{\cdot}0181$
(*b*) from the index *v*, $0{\cdot}0237 \pm 0{\cdot}0162$.

By all criteria, therefore, it is best to employ *v* as a criterion of response. The fiducial limits of error, using *v* ($P = 0{\cdot}95$), for an assay of the first and third concentrations against the other two are 97·9–114·0 %, mean estimate 105·6 %. (The correct answer is, of course, 100 %.)

Further tests were made in which known dilutions (but unknown to the operator) of preparations of hyaluronidase were tested against the parent solutions. Since no significant mean square was associated with interaction, each concentration was tested only once with each viscosimeter, in order to determine the accuracy of assays using a small number of individual determinations of flow-time. The results of these tests are shown in Tables 4 and 5.

There is rather a large difference between the mean squares for interaction, used as error, in these two tests. The difference is significant at the 5 % level, but not at the 1 % level. The apparent failure completely to eliminate differences between instruments in the first test (Table 4) is more likely due to a depressed error mean square in this test than to a real failure of elimination, as the error mean square is lower than in the other similar tests here reported. There is also a significantly better agreement between the slopes of the two dose/response lines in Table 4 than could be expected by chance; again the significance is not high ($P > 0{\cdot}95$, $< 0{\cdot}99$). Otherwise, these two further tests are completely satisfactory; the internal evidence from each shows that a valid assay with quite low limits of error has been performed. From the trial test reported in Tables 1–3, it would have been predicted that an assay with three observations at two concentrations of each of two substances should have an error of about ± 14 % ($P = 0{\cdot}95$), whereas we find errors ($P = 0{\cdot}95$) of about ± 10 and ± 20 % in the two trials, and deviations from the known potency ratio of 3 and 4 %.

Table 4. *Analysis of variance for a test of two samples of hyaluronidase with a potency ratio of* 0·292

Source of variation	Degrees of freedom	Mean square	*F*
Differences between samples*	1	60066	341†
Slope of combined dose/response line	1	89960	505†
Departure from parallelism	1	0·083	0·00047‡
Differences between viscosimeters	2	1146	6·4‡
Interaction (error)	6	178·4	—

* Estimated potency ratio = 0·284, limits ($P = 0{\cdot}95$) 0·257–0·313, or 90·7–110·3 % of the mean estimate.
† $P > 0{\cdot}99$.
‡ $P > 0{\cdot}95$, $< 0{\cdot}99$.

Table 5. *Analysis of variance for a test of two samples of hyaluronidase with a potency ratio of* 0·70

Source of variation	Degrees of freedom	Mean square	*F*
Differences between samples*	1	27075	30·6†
Slope of combined dose/response line	1	82336	92·7†
Departure from parallelism	1	33·3	0·038
Differences between viscosimeters	2	506·3	0·57
Interaction (error)	6	886·8	—

* Estimated potency ratio = 0·674; limits ($P = 0{\cdot}95$) 0·551–0·823, or 82·0–122·4 % of the mean estimate.
† $P > 0{\cdot}99$.

Analysis of several subsequent assays has yielded results fully in agreement with the above findings, the errors ranging from ± 3 to ± 16 % ($P = 0{\cdot}95$).

SUMMARY

1. Some of the fundamental principles involved in the viscosimetric assay of hyaluronidase have been investigated.

2. As a result, a method of assay has been devised depending upon the fact that, within certain prescribed limits, the fall in the flow-time through an Ostwald viscosimeter of a buffered hyaluronate-hyaluronidase mixture incubated for a fixed time is proportional to the logarithm of the concentration of the enzyme.

3. Satisfactory assays which have standard errors of less than 10% can be made with a total of twelve readings.

Our thanks are due to Dr S. J. Folley for providing the bull semen from which the standard hyaluronidase preparation has been made. We are also indebted to Dr H. J. Rogers and Dr I. W. Rowlands for invaluable advice.

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The Action of Sulphites on the Cystine Disulphide Linkages of Wool

5. THE EFFECT OF SOME CHEMICAL MODIFICATIONS OF THE WOOL ON THE MAGNITUDE OF THE BISULPHITE-REACTIVE FRACTION ($A+B$) AND THE RELATIVE MAGNITUDES OF SUBFRACTIONS A AND B

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Elsworth & Phillips (1938, 1941) showed that approximately half of the cystine disulphide-S of wool reacted with solutions of $NaHSO_3$ to give thiol and *S*-cysteine-sulphonate groups according to the reaction:

$$R'.CH_2SSCH_2.R'' + NaHSO_3 \rightleftharpoons R'.CH_2SH + NaOSO_2SCH_2.R'',$$

where R' and R'' represent polypeptide chains. Middlebrook & Phillips (1942) found that this reactive fraction of the disulphide-S could be divided into two subfractions: subfraction A which was readily reversed to disulphide on rinsing the bisulphited wool with water, and subfraction B which gave thiol and *S*-cysteine-sulphonate groups which were stable to water-rinsing.

In an endeavour to elucidate the cause of this difference in the behaviour of subfractions A and B, we have examined a number of chemically modified wools. Whilst we have not discovered any method of modifying the wool which causes more of the total cystine-S to react with $NaHSO_3$, we have found that any treatment which blocks the free carboxyl groups of the combined glutamic and aspartic acids, produces an increase in the number of thiol and *S*-cysteinesulphonate groups stable to water rinsing (subfraction B) and a corresponding decrease in the water-labile subfraction A.

METHODS

The chemically modified wools were prepared, as described below, from the sample of wool (64's combed merino) used by Lindley & Phillips (1945). All analyses are calculated on the weight of the anhydrous wool.

Deaminated wool. The wool (10 g.) was immersed for 2 days in a solution prepared by mixing 80% (w/v) $NaNO_2$ (2 vol.) with glacial acetic acid (1 vol.), washed for 2 days in running water, dried and conditioned by exposure to a controlled atmosphere of 21° and 70% relative humidity until it attained constant weight. This method deaminates the lysine ϵ-amino-N and in addition an indefinite number of terminal arginine amino-N groups (Speakman & Stott, 1934; Rutherford, Harris & Smith, 1937).

Acetylated wools. These were obtained by (*a*) boiling, under reflux, for 30 min. with excess acetic anhydride, (*b*) by immersion overnight in acetic anhydride at room temperature.

Methylated wools. These were prepared by the methods described by Blackburn, Carter & Phillips (1941), Blackburn & Phillips (1944). Prior to conditioning, all the methylated wools were rinsed thoroughly in distilled water to remove buffer salts.

O-Methyl was determined by Baernstein's method (1932, 1936). Pregl's method (1924) was used for *N*-methyl, but the methyl iodide evolved was determined as in the *O*-methyl determinations. Neither the use of gold chloride nor fuming HI was found advantageous (Edlbacher, 1918). Preliminary investigations showed that in agreement with

observations on casein by Burns (1914) and Geake & Nierenstein (1914), untreated wool gave an apparent *N*-methyl content of 0·32%. Burns (1914) attributed the apparent *N*-methyl content of unmethylated casein to the decomposition of glycine and alanine to methylamine and ethylamine on heating with HI at the elevated temperatures used in Pregl's method. We were unable to devise conditions which would give satisfactory results for the *N*-methyl content of sarcosine-HCl. The *N*-methyl contents of the methylated and untreated wools were therefore carried out under as nearly as possible identical conditions. The wools were first hydrolyzed in boiling HI under reflux for 3 hr. and the hydrolysate then heated at 290–300° for 1 hr.

Amino-N of wool. An apparatus was designed for use with the Van Slyke (1927) manometric gas analysis apparatus. Doherty & Ogg (1943) have since described a similar apparatus and technique, except that they used wool which had been ground in a ball mill, whereas we used intact wool. Our results agree with theirs.

Treatment of modified wools with sodium bisulphite. The wools were treated with 100 times their weight of 30% (w/v) $NaHSO_3$ for 17 hr. To wet them thoroughly, the samples were submerged in the $NaHSO_3$ by a glass sinker, the space above the solution was exhausted and then air was re-admitted. Fraction $(A+B)$ of the disulphide-S was determined by hydrolyzing 0·2 g. of the bisulphited wool, after it had been squeezed to remove the excess $NaHSO_3$ solution, in 20 ml. of 5N-HCl for 4 hr., and estimating the cysteine and cystine-S liberated by Shinohara's methods (1935*a*, *b*) using a Spekker absorptiometer. Two methods of rinsing the wool were used before the cysteine liberated from subfraction *B* was determined. In our earlier experiments, the bisulphited wools were rinsed during ½ hr. in four changes of tap-water of pH 9 (Lindley & Phillips, 1945) but in later experiments they were rinsed by the following procedure of Carter, Middlebrook & Phillips (1946): after rinsing the bisulphited wool (0·2 g.) for 10 min. in 250 ml. of distilled water, it was immersed overnight in 2l. of distilled water. The rinsed wool was hydrolyzed in 10 ml. 5N-HCl for 4 hr. and the cysteine and cystine contents of the hydrolysate determined.

RESULTS

Fraction $(A+B)$ and subfraction B of the combined cystine-S of methylated wools

The CH_3 contents, disulphide-S, fraction $(A+B)$ and subfraction *B* of the cystine-S of untreated wool and of wool methylated by three different reagents are given in Table 1. The CH_3 contents were unaltered by bisulphiting, except in the case of the wool methylated with methyl sulphate, the CH_3 content falling to 0·86%.

Blackburn *et al.* (1941) have produced evidence that during methylation of wool with methyl bromide and methanol-acetic anhydride only the carboxyl groups of the combined glutamic and aspartic acids are esterified. Methyl sulphate also esterifies carboxyl groups, but in addition *O*-methylates some other centre. These additional CH_3 groups, which are possibly attached to peptide linkages (Blackburn *et al.* 1941; Blackburn, Middlebrook & Phillips, 1942; Blackburn & Phillips, 1944) are removed easily either by steam or alkaline buffers. It now appears that $NaHSO_3$ is a useful reagent for removing selectively these CH_3 groups.

Table 1. *The disulphide-S of fraction $(A+B)$ and of subfraction B of methylated wools*

Methylating agent	CH_3 (%)	Di-sulphide-S (%)	Fraction $(A+B)$ (%)	Subfraction B* B' (%)	Subfraction B* B'' (%)
Untreated	0·0	3·10	1·84	1·02	0·58
Dimethyl sulphate	1·44	2·86	1·74	1·60	1·46
Methyl bromide	0·80	2·60	1·65	1·30	1·22
Methanol-acetic anhydride	0·47	3·02	1·72	1·52	0·86

* B' stable to rinsing in tap-water of pH 9 (Lindley & Phillips, 1945); B'' stable to rinsing in distilled water (Carter *et al.* 1946).

The results given in Table 1 show that esterification of the free carboxyl groups does not affect greatly the amount of disulphide-S which reacts with bisulphite, i.e. fraction $(A+B)$ is substantially unchanged, but it does increase the water-stable subfraction *B* considerably at the expense of water-labile subfraction *A*. To pursue the investigation further, wools of increasing CH_3 contents were prepared by each method of methylation. The initial CH_3, disulphide-S and fraction $(A+B)$ of these wools are given in Table 2, whilst Fig. 1 shows graphically the relationship between subfraction *B* and the CH_3 content of the methylated and bisulphited wools. Subfraction *B* was determined after these wools had been rinsed in distilled water (Carter *et al.* 1946). Two lines are shown in Fig. 1: both have a slope corresponding to four CH_3 groups to each disulphide bond (i.e. 2 S atoms) of subfraction *B*. The upper line is drawn through the point 0·0% CH_3 and 0·58% S, these values corresponding to those of unmethylated, bisulphited wool after rinsing in distilled water. This line provides an excellent fit for the results on the wools methylated with methyl sulphate and a fairly good approximation for the wools methylated with methyl bromide. The lower line has the same slope, but cuts the axis at 0·42% S which is the value of the subfraction *B* of wool acetylated with cold acetic anhydride.

The simple conclusion seems therefore to emerge from these experiments that the difference in stability towards water of the thiol and *S*-cysteine sulphonate groups of subfraction *A* and *B* is due to the presence of free carboxyl groups of glutamic and aspartic acids in the neighbourhood of subfraction *A*.

Table 2. *The initial* CH_3, *disulphide*-S *and fractions* $(A+B)$ *of methylated wools*

Methylating agent	CH_3 (%)	Disulphide-S (%)	Fraction $(A+B)$ (%)
Dimethyl sulphate	0·31	3·10	1·87
	0·46	3·16	1·82
	0·73	3·04	1·80
	0·99	3·17	1·86
	1·94	2·78	1·75
Methyl bromide	0·15	3·02	1·74
	0·23	2·92	1·80
	0·34	2·94	1·75
	0·51	2·82	1·75
	0·63	2·77	1·73
	0·81	2·79	1·79
	1·29	2·72	1·74
Methanol-acetic anhydride	0·14	3·11	1·66
	0·72	3·09	1·79
	0·86	3·06	1·83

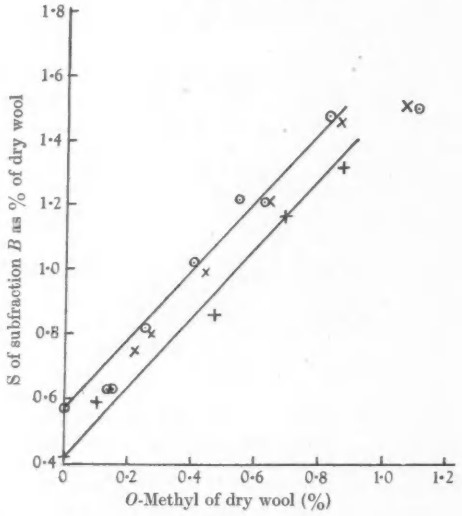

Fig. 1. The relationship between subfraction *B* and degree of methylation of various methylated wools. ⊙ Wool methylated with methyl bromide. × Wool methylated with methyl sulphate. + Wool methylated with methanol-acetic anhydride.

The N-methyl of methylated wools

Besides esterifying free carboxyl groups, there is some evidence (Blackburn *et al.* 1941) that the methylating agents used to prepare the methylated wools also cause some *N*-methylation. Attempts were made to measure the degree of *N*-methylation of the wools, since it may have influenced the relative water-stabilities of the thiol and *S*-cysteinesulphonate groups derived from subfractions *A* and *B*.

(*a*) *Direct estimations.* The *N*-methyl contents of the untreated and methylated wools (other analyses of which are given in Table 1) as determined by Pregl's method are given in Table 3.

Table 3. *The N-methyl contents of methylated wools*

Methylating agent	*N*-methyl (%)
(Untreated)	0·32
Dimethyl sulphate	0·64
Methyl bromide	0·73
Methanol-acetic anhydride	0·74

(*b*) *Indirect determination by estimations of terminal* NH_2 *groups.* (i) *ε-Amino*-N *of lysine.* Rutherford *et al.* (1937) showed that it is possible to estimate the ε-amino-N of the combined lysine in

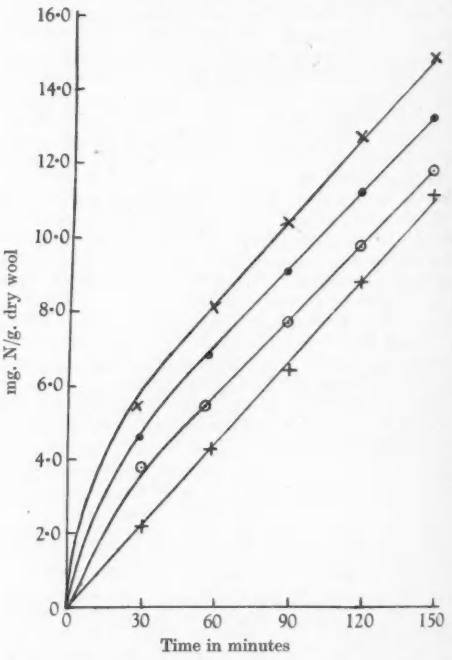

Fig. 2. The rate of liberation of nitrogen from methylated wools on treatment with nitrous acid. ● Untreated wool. + Wool methylated with methanol-acetic anhydride. ⊙ Wool methylated with methyl bromide. × Wool methylated with methyl sulphate.

wool from the rate of liberation of N_2 on treating wool with nitrous acid: *N*-methylation of this amino-acid should be detectable therefore by a decrease in the amino-N.

Fig. 2 shows the rate at which N_2 was liberated, when the untreated and methylated wools of Table 1

were treated with nitrous acid. Extrapolation of the straight line portion of the curves back to zero time gives the following values for the amino-N of the wools: untreated wool, 0·31 %; methyl bromide-treated wool, 0·20 %; dimethyl sulphate-treated wool, 0·37 %; methanol/acetic anhydride-treated wool, 0·00 %.

Some methylation of the ϵ-amino-N of lysine thus occurs with methyl bromide, but no explanation can be offered for the increase in the amino-N of the wool methylated with methyl sulphate, except that some slight hydrolysis may have taken place during this rather prolonged methylation. The disappearance of amino-N from the wool when methylated with methanol/acetic anhydride is not surprising, since it is probable (Blackburn & Phillips, 1944) that this reagent methylates and acetylates respectively the carboxyl and amino-groups of opposed side-chains. *N*-Acetylation rather than *N*-methylation is therefore the more probable explanation of the disappearance of the amino-N in this instance.

(ii) *Arginine.* Arginine is the other amino-acid in wool, with a terminal amino-group which might be methylated. Arginine estimations were therefore made on untreated wool and a wool (CH_3, 2·0 %) which had been methylated with methyl sulphate. Expressed as percentage of total N, the arginine-N contents of the wools by Vickery's (1940) method were: untreated wool, 18·7; methylated wool, 18·9. Although the arginine flavianate isolated from the methylated wool appeared to be pure, it was converted into the hydrochloride (Pratt, 1926) and the purity was tested chromatographically (Consden, Gordon & Martin, 1944). It was found that whereas the arginine hydrochloride obtained from the flavianate isolated from the untreated wool behaved normally, that from the flavianate isolated from the methylated wool did not give the usual colour when heated with ninhydrin. A mixture of the two hydrochlorides also gave no colour with ninhydrin, although both gave a very strong Sakaguchi reaction. The arginine from the methylated wool thus appeared to contain an impurity (possibly a *N*-methyl derivative of arginine) which inhibited the ninhydrin reaction and could not be separated readily from arginine by partition chromatography using the filter-paper technique.

Fraction (A + B) and subfraction B of the combined cystine-S of acetylated and deaminated wools

The S distributions of the wools which were acetylated and deaminated to modify their basic side-chains and then bisulphited are given in Table 4.

From Table 4 it will be seen that cold acetylation does not affect the magnitude of fraction $(A+B)$, although subfraction B is decreased slightly. On the other hand, acetylation with boiling acetic anhydride and deamination both affect the reactivity of the combined cystine-S: hot acetylation causes fraction $(A+B)$ to fall from 1·84 to 1·15 %, whilst deamination causes it to fall to 1·07 %. To some extent both these changes are due to the destruction of disulphide-S. In addition, it is probable that hot acetylation and deamination, besides altering the terminal amino-groups, also attack other centres in the wool.

Table 4. *The disulphide-S and subfractions A and B of acetylated and deaminated wools*

Treatment	Di-sulphide-S (%)	Fraction $(A+B)$ (%)	Sub-fraction A (%)	Sub-fraction B* (%)
Acetylated (cold)	3·10	1·79	1·36	0·43
Acetylated (hot)	2·70	1·15	1·13	0·02
Deaminated	2·70	1·07	0·58	0·49

* Stable to rinsing in distilled water (Carter *et al.* 1946).

DISCUSSION

Astbury (1942) has suggested that approximately equal numbers of polar and non-polar side-chains of wool are disposed on opposite sides of the polypeptide chains. Hence successive chains in a polypeptide grid may be separated alternately by polar and non-polar side-chains. Carter *et al.* (1946) have pointed out that such a disposition of side-chains might explain why the combined cystine of wool falls into two approximately equal fractions: fraction $(A+B)$ which may cross-link the main-chains separated by polar side-chains and fraction $(C+D)$ which may cross-link the main-chains separated by non-polar side-chains.

The present work has demonstrated that the relative magnitudes of subfraction A and B can be changed by the esterification of the free carboxyl groups of the combined glutamic and aspartic acids. Probably the success attending these experiments has been due to the mild and selective methods by which the carboxyl groups were esterified. Although our experimental evidence is less conclusive, the methylation or acetylation of the terminal amino-groups of lysine and arginine is without systematic effect. Hot acetylation and prolonged treatment of wool with nitrous acid would appear to attack too many different side-chains to bring about simple changes in the reactivities of subfractions A and B.

Since the chemical reactivities of these two subfractions are closely similar and tend to become identical when the free carboxyl groups are converted to carbomethoxyl groups, it is possible that whilst free carboxyl groups predominate in the

environment of subfraction *A*, amide groups predominate in the environment of subfraction *B*. On Astbury's (1942) calculations there are enough dicarboxylic acid residues and amide groups to provide each disulphide group of subfraction *A* with an environment containing 6 carboxyl groups and each disulphide group of subfraction *B* with an environment containing 6 amide groups. At present, it is inadvisable to pursue this suggestion further, since the available analyses of the glutamic and aspartic acids and their amides in wool are too variable.

Attempts were made to remove the amide groups of wool by hydrolysis with boiling 0·1 N-HCl and to examine the reaction of the deaminated wool with sodium bisulphite. To lower the amide-N of the wool from 1·31 to 0·80 %, the wool had to be boiled for 8 hr. The wool then tended to disperse in the bisulphite and made quantitative interpretation of the results impossible. It was observed, however, that the partially deamidated wool gave qualitatively the same reaction as normal wool with sodium bisulphite: fraction $(A+B)$ was less than the total cystine-S and was divisible into subfraction *A* and *B* by water rinsing.

The observed influence of esterification on the relative magnitudes of subfractions *A* and *B* has a bearing on the question whether the different reactivities of the cystine subfractions arise from differences in the accessibility of the subfractions to the reagents we have used. In the first place, such differences in accessibility might arise from variation in the degree of 'crystallinity' of different parts of the fibres. One would expect that the variation in chemical reactivity to which this might give rise would tend to be eliminated by increasing the severity of the treatments. Experimentally, however, when the treatments are made more severe, the additional cystine-S that is caused to react undergoes a different type of reaction. For example, the more reactive fraction $(A+B)$ is converted into thiol and *S*-cysteinesulphonate groups by bisulphite and into lanthionine by alkalis: fraction $(C+D)$, on the other hand, when caused to react by increasing the severity of the treatments, is converted into combined α-aminoacrylic acid by both these reagents.

Such differences in the type of reaction which takes place may arise from variations in the restrictions imposed on the movement of groups by the interactions of neighbouring side-chains. For example, the ease of reversibility of the reaction between the combined cystine of fraction $(A+B)$ and sodium bisulphite may depend on the ease with which the thiol and *S*-cysteinesulphonate groups that are produced can move apart. This in turn may depend on the nature of the neighbouring side-chains. A possible explanation of the difference between subfractions *A* and *B* may be that whereas the neighbouring side-chains of subfraction *A* give salt-linkages, those of subfraction *B* give cross-linkages of a less stable character. On such assumptions, the thiol and *S*-cysteinesulphonate groups of subfraction *A* might be held closer together than those derived from subfraction *B*.

SUMMARY

1. A study has been made of the reactivity of the cystine-S of chemically modified wools.

2. Esterification of the free carboxyl groups of the combined glutamic and aspartic acids, whilst it does not increase the magnitude of the bisulphite-reactive fraction $(A+B)$, causes the thiol and *S*-cysteinesulphonate groups produced from subfraction *A* to become stable to water-rinsing like those produced from subfraction *B*.

3. Hot acetylation and deamination by prolonged treatment with nitrous acid do not cause systematic changes in the magnitudes of subfractions *A* and *B*.

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The Determination of Iron in Biological Material

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In the course of investigations on protein hydrolysates used for preparing culture media, it was found that no satisfactory method for determining small quantities of iron (0·5–10 μg./ml.) in such material existed. Wet ashing was reputed to give reliable results with biological material (Jackson, 1938; Thorp, 1941), but this method is time-consuming and the neutralization of the residual acid after digestion introduces adventitious iron, resulting in large and troublesome blanks. The alternative process of dry ashing in an electric muffle furnace offers a convenient and at first sight complete solution, but experience proves that this method introduces an unexpected chemical problem concerning iron in complex combination. It was found that recoveries of iron from such ashes were variable and low, and in addition, combination with $\alpha\alpha'$-dipyridyl to develop a pigment suitable for colorimetric measurement was slow and incomplete.

The determination of small quantities of iron in dry ashes of material with a high P : Fe ratio has been studied by many workers, and the diverse methods which have been suggested to overcome the difficulties, attributed to complex phosphate formation, reflect the present unsatisfactory situation (Elvehjem & Hart, 1926; Elvehjem, 1930; Jackson, 1938; Howe, 1943, 1944; Bandemer & Schaible, 1944; Stugart, 1931; Koenig & Johnson, 1942). Methods which involved much manipulation after ashing, as, for instance, the complete removal of phosphates (Elvehjem & Hart, 1926), were considered unsuitable, since the main reason for using dry ashing was the low level at which blanks could be kept. The simpler methods usually involved boiling with acid or alkali for varying times (Howe, 1944; Stugart, 1931). An iron complex resistant to this procedure, presumably similar to that reported in ashes of tomato and spinach (Jackson, 1938), was found to be present in ashes of protein hydrolysates. The present communication reports a procedure overcoming this interference and the development of a simple method of good precision for estimating iron.

EXPERIMENTAL

The method as finally developed consists in (*a*) dry ashing the sample, (*b*) precipitating the iron present as sulphide by means of ammonium sulphide solution and (*c*) dissolving the precipitated ferrous sulphide in acid in the presence of $\alpha\alpha'$-dipyridyl. The resulting red colour is measured in an absorptiometer. The iron sulphide is not separated from the ash solution, and all reactions are carried out in the crucible in which the sample has been ashed. Blanks can therefore be kept low because of the very small amount of manipulation involved.

The method of ashing has been found to have a marked effect on the reproducibility of the method, and the one adopted is a modification of that used by Klumpp (1934) who damped his material with H_2SO_4 before ashing to prevent loss of iron. The H_2SO_4 acts as an effective ashing aid, and the white fluffy ashes so obtained dissolve readily and give no carbon residue of any kind.

When the search for a suitable method for determining iron was started, potassium thiocyanate was used as the chromogenic agent. This was found unsuitable owing to the necessity for strict pH control, and the poor stability of the colour formed limited the number of determinations able to be carried out at one time. $\alpha\alpha'$-Dipyridyl was found to be a more suitable reagent, since the red-coloured complex it forms with ferrous ions is stable for a period of months (Thorp, 1941; Moss & Mellon, 1942) and its colour is not affected over the pH range 2·5–8·5 (Moss & Mellon, 1942).

It was observed that dry ashing of protein hydrolysates followed by HCl and sodium sulphite treatment for the reduction of iron gave non-reproducible results. Experimental study led to the conclusion that dry ashing resulted in a ferric complex stable to acid hydrolysis. A difficulty of the same kind was reported by Jackson (1938), who introduced the use of ammonium sulphide. This enabled ferrous sulphide to be recovered for subsequent treatment. While the application of this method in the original form, or modified by the use of adsorbents such as calcium phosphate, proved unsuccessful in our case, the use of ammonium sulphide for the purpose of bringing ferrous iron and $\alpha\alpha'$-dipyridyl into quantitative reaction enabled the following experimental method to be successfully applied.

METHOD

Apparatus

Ashing can be carried out in platinum or silica dishes or in Royal Worcester porcelain crucibles, provided the latter are well cleaned with chromic acid before use.

Measurement of colour

A Spekker absorptiometer with 1 cm. cells and Wratten 61 green filters was used for all the colour measurements, but any other suitable instrument can be used.

Reagents

All reagents are A.R. and the distilled water is redistilled from glass.

Acids. N-H_2SO_4 and N and 0·1 N-HCl. The hydrochloric acid solutions are prepared from glass-distilled constant-boiling (20%) HCl.

Ammonium sulphide solution. Freshly prepared for each set of determinations by bubbling H_2S through concentrated ammonia solution (sp.gr. 0·880 A.R.) for 2–5 min. The final solution should not be more than faintly yellow. A.R. ammonium sulphide is not suitable, as it tends to deposit sulphur under the experimental conditions. This deposit is most difficult to remove and makes estimation with the photoelectric absorptiometer impossible.

αα′-Dipyridyl. A 1% (w/v) solution in Fe-free 0·1 N-HCl This solution is rejected as soon as it shows more than a slight red colour.

Sodium sulphite. A 20% (w/v) solution of $Na_2SO_3.7H_2O$ prepared just before use.

pH 4·6 *buffer.* This is made by mixing 200 ml. of M-sodium acetate with 100 ml. N-HCl (iron-free) and making to 1 l.

Standard Fe *solution.* This is made from pure iron wire or ferric ammonium sulphate. A primary standard containing 100 μg. Fe/ml. is conveniently prepared by dissolving 0·8636 g. of $Fe(NH_4)(SO_4)_2.12H_2O$ in water, adding 100 ml. of N-HCl and making up to 1 l.

Standard curve

For the method described a calibration curve was prepared for the Spekker absorptiometer over the range 0–100 μg. Fe/10 ml. Colour development of standards was carried out by reducing with sodium sulphite and HCl in the presence of αα′-dipyridyl (Thorp, 1941). The use of sodium sulphite and acid for reduction was considered preferable to ammonium sulphide as reduction was complete, the acid and sulphite buffered within the working pH range of αα′-dipyridyl and the necessity for redissolving a ferrous sulphide precipitate was avoided. The standard iron solution was diluted so that it contained 20 μg. Fe/ml. Suitable quantities to cover the range 0–100 μg. Fe were pipetted into 10 ml. graduated centrifuge tubes and distilled water added to give a volume of 5 ml. Then 0·5 ml. 20% sodium sulphite solution, 0·5 ml. N-HCl and 0·25 ml. αα′-dipyridyl solution were added. The contents of each tube were well mixed and left for 15 min., before making to 10 ml. and reading in the absorptiometer. Tubes showing any cloudiness were centrifuged before colour measurement, but in most cases this was not found necessary.

Procedure

Weigh or pipette a suitable quantity of material into a platinum, silica or Royal Worcester crucible. For hydrolysates containing between 0·5 and 10 μg. Fe/ml., 5–10 ml. has been found satisfactory. Add 2·5 ml. of N-H_2SO_4/10 ml. (or 10 g.) and evaporate on a water-bath to a consistency suitable for ashing. Protect samples on the water-bath from aerial contamination by shielding them with a sheet of glass. Ash at 500°. Cool the ash, add 1·0 ml. of N-HCl and take to dryness on the water-bath. Cool, add 5 ml. of distilled water and 0·25 ml. of ammonium sulphide solution. Mix with swirling and again dry and cool. Add 2 ml. water, 0·5 ml. of αα′-dipyridyl solution and 0·5 ml. N-HCl. Place the crucible on the water-bath just off the boil and leave 5 min. with occasional swirling to assist complete solution of the ferrous sulphide. It is most important that complete solution is achieved, since this has been found to be the main cause of bad duplication of results. A black residue is sometimes seen if much copper is present and is centrifuged off at a later stage. Add 5 ml. of pH 4·6 buffer to bring the reaction of the solution within the range for maximum colour development. Cool and transfer the contents of the crucible to a graduated centrifuge tube. Make up to 10 ml. and centrifuge to remove any suspended matter likely to interfere with colour matching. Read in the absorptiometer

RESULTS

Sixteen 10 ml. samples of a papain digest of horse muscle were analyzed and the following results obtained: 62·0, 63·8, 60·7, 61·9, 59·2, 61·4, 62·1, 62·0, 62·1, 63·8, 61·5, 59·9, 62·8, 61·0, 59·7, 60·5 μg. Fe/10 ml. hydrolysate. The mean was 61·5 μg. Fe/10 ml., and the standard deviation from the mean 1·3 μg. Fe/10 ml., giving a coefficient of variation of 2·1%.

Table 1. *Recoveries of iron from papain digest of horse muscle and from milk*

	Fe found (μg./5 ml.)		
	Papain digest	Papain digest +10 μg. Fe	Papain digest +20 μg. Fe
	31·0	40·0	50·0
	31·0	41·0	52·0
	30·0	41·0	49·0
Average	30·7	40·7	50·3
Fe recovered (%)		100·0	98·0

	Fe found (μg./10 ml.)		
	Milk	Milk +10 μg. Fe	Milk +20 μg. Fe
	4·8	15·0	25·0
	4·5	15·5	25·0
	4·7	14·5	25·0
Average	4·7	15·0	25·0
Fe recovered (%)		103·0	101·5

In Table 1 are given the results of recovery experiments on 5 ml. samples of papain digest of horse muscle to which were added 10 and 20 μg. of Fe and a similar series with 10 ml. of fresh raw cows' milk. The iron was added as a solution of ferric ammonium sulphate before ashing, and ashings were in triplicate.

DISCUSSION

The method described is based on two observations. The first, made by Jackson (1938), is that small amounts of iron can be quantitatively precipitated as sulphide from ashes of biological material with a high P : Fe ratio. The second, that the ferrous sulphide so precipitated can be redissolved without separation from the interfering ions present, and combined quantitatively and preferentially with $\alpha\alpha'$-dipyridyl, was made while trying to apply Jackson's methods to ashes of protein hydrolysates. These ashes contain an iron complex very resistant to acid hydrolysis. It is therefore unlikely to be iron pyrophosphate, which can be hydrolyzed by acids (Jackson, 1938; Howe, 1944). The nature of the complex is not known, but it may be significant that iron metaphosphates can be formed at 200–300° and are stable up to 800° (Brasseur, 1944). Direct experimental confirmation of the presence of the metaphosphate ion is difficult; its solutions, however, are known to have marked tendencies to sequester multivalent ions. After reduction and precipitation of iron as sulphide, one or more possible interfering ions are left in solution. It seemed that introduction of $\alpha\alpha'$-dipyridyl and re-solution of the iron would be likely to lead to the preferential formation of stable ferrous dipyridyl rather than any of the unwanted ferric complexes. Any tendency for the iron to revert to the ferric state would be checked by the nascent H_2S evolved from the dissolving ferrous sulphide. This appeared to be the case.

SUMMARY

1. A method for determining iron in biological materials with a high P : Fe ratio is described. It is applicable over the range 0·5–10 μg. Fe/ml. All analytical manipulations, except the final centrifuging before colour reading, are performed in the crucible in which the sample has been ashed. Blanks are therefore kept at a minimum.

2. The method has proved satisfactory with protein hydrolysates and cows' milk and may be suitable for other materials where phosphate interference is met.

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The Excretion of *l*(−)-Tyrosine in Urine

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Estimation of the *l*(−)-tyrosine content of urine is of value in the assessment of hepatic disease (Lichtman, 1934) and in the study of *l*(−)-tyrosine metabolism. The methods hitherto available lack the necessary sensitivity and specificity for the measurement of normal and pathological tyrosinuria. Thus the tyrosinase method of Lichtman & Sobotka (1929) fails to detect the tyrosine present in normal urine, and Millon's reaction lacks specificity.

In the present communication a method is described which estimates only *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine and is sufficiently sensitive to measure the *l*(−)-tyrosine content of normal urine. The material estimated is referred to throughout this paper as *l*(−)-tyrosine because the presence of *l*-3:4-dihydroxyphenylalanine in normal urine is considered unlikely. The possibility that a mixture of the two substances has been estimated has not however been excluded. Data have been obtained for a small series of normal men and women and a rough assessment made of the *l*(−)-tyrosine output of a few normal domestic animals. The output of *l*(−)-tyrosine has also been measured in pregnancy and in a few pathological conditions.

Evidence for the occurrence of tyrosine in normal urine was obtained by Embden & Reese (1906), although a pure product was not isolated. Pouchet (1880) is quoted by Neubauer & Huppert (1910) as

having detected tyrosine in normal urine, but the original publication is not accessible. Rosenbloom & Gardner (1914) reported the occurrence of tyrosine crystals in the urine of a normal pregnant woman.

METHODS

l(−)-Tyrosine was estimated by submitting 2·0 ml. of urine, or urine concentrate, to the action of a specific preparation of *l*(−)-tyrosine decarboxylase (Epps, 1944) and measuring the CO_2 evolved in a Warburg manometer as in the analysis of protein hydrolysates described by Gale (1945). This enzyme decarboxylates *l*(−)-tyrosine and *l*-3:4-dihydroxyphenylalanine specifically (Epps, 1944; Gale, 1940).

Preparation of urine. If concentration of the urine is not required, the acidity of the urine is adjusted to pH 5·5 with acetic acid. In the case of normal urine the *l*(−)-tyrosine content is too low for direct estimation, and the following procedure for concentration of the urine is used. Urine (200 ml.) and crystalline oxalic acid (6 g.) are concentrated by vacuum distillation almost to dryness. The oxalic acid maintains a pH low enough to retain *l*(−)-tyrosine in solution and prevent hydantoin formation (Dakin, 1910), and precipitates much urea as urea oxalate. The concentrate is washed into a weighed flask; its volume and the washings should not exceed about 30 ml. The preparation is then filtered by suction and a sample of the total fluid component, determined gravimetrically, brought to pH 5·5 with a measured volume of 10N-NaOH. The dilution involved in this neutralization is noted and allowed for in the calculation of the *l*(−)-tyrosine content.

Manometric estimation. The concentrate (2·0 ml.) at pH 5·5 is measured into the main chamber of the Warburg manometer cup and 0·2 ml. of 2·2M-citrate buffer of pH 5·5 added. Into the side arm is measured 0·8 ml. of a 2·5% (w/v) suspension of enzyme preparation in 20% (w/v) Na_2SO_4 buffered to pH 5·5 with 0·2M-citrate. After temperature equilibration, the enzyme is tipped into the main chamber and readings taken until a constant value is obtained. A control unit is set up in which the enzyme suspension is replaced by buffered Na_2SO_4, and its readings are deducted from the test readings. The weight in mg. of *l*(−)-tyrosine in the manometer cup is $0{\cdot}00844 \times (\mu l.$ of CO_2 evolved). This factor allows for the finding of Gale (1945) that the manometric output of CO_2 is 96% of the theoretical value. From this finding the *l*(−)-tyrosine content of the urine can be readily calculated. When unconcentrated urine, or *l*(−)-tyrosine standards are analyzed the enzyme is suspended in 0·2M-citrate buffer instead of buffered Na_2SO_4. The 20% (w/v) Na_2SO_4 and the 2·2M-citrate buffer are supersaturated solutions at room temperature and should be warmed sufficiently to secure complete solution immediately before use.

RESULTS

Control readings in absence of l(−)-tyrosine. The tipping of the buffered enzyme preparation into the 0·2M-citrate buffer used in these experiments causes no manometric change. When urine concentrate is used in the main chamber a large positive blank reading is obtained on tipping in 0·8 ml. of 0·2M-buffer. This blank can be shown not to be due to liberation of CO_2 by arranging for CO_2 absorption in the centre cup, when the blank reading is unchanged. If the buffer in the side arm contains 20% (w/v) of Na_2SO_4, a very small blank reading is obtained with most urine concentrates, and this concentration of Na_2SO_4 was therefore used in the enzyme suspension when analyzing samples of concentrated urine.

Effect of urea and of Na_2SO_4 upon decarboxylase activity. When *l*(−)-tyrosine equivalent to 74 μl. of CO_2 was treated with decarboxylase in the presence of 20% (w/v) of urea, 84% inhibition was observed. A similar experiment in the presence of 20% (w/v) Na_2SO_4, instead of the urea, showed no inhibition, as would be expected from the absence of salt effect up to 33% saturation with $(NH_4)_2SO_4$ found by Epps (1944). Thus 20% Na_2SO_4 can be safely used to suspend the enzyme in the side arm.

Recovery of l(−)-tyrosine added to urine. Solutions of *l*(−)-tyrosine in normal urine were prepared over a range of concentration that might be encountered in clinical work (Lichtman, 1934). These solutions gave on analysis the recoveries shown in Table 1.

Table 1. *Recovery of l(−)-tyrosine added to urine*

Nature of urine	*l*(−)-Tyrosine added (mg./100 ml.)	Recovery of added *l*(−)-tyrosine: Observations (%)	Recovery of added *l*(−)-tyrosine: Mean (%)
Normal (not concentrated)	57·1	97, 99	98
Normal (not concentrated)	40·0	100, 103	102
Normal (not concentrated)	19·0	99, 100	100
Normal (not concentrated	5·0	105	
Normal (not concentrated)	3·0	104	
Normal (not concentrated)	2·0	120, 161	141
Urine containing bile pigment	30·0	98	
Urine preserved with toluene	30·0	99	
Normal urine (concentrated)	3·0	95, 101	98
Normal urine (concentrated)	2·5	97, 99	98

Although good recovery was obtained without concentration of the urine with additions of *l*(−)-tyrosine as low as 3·0 mg./100 ml., yet it is advisable to concentrate the urine whenever the *l*(−)-tyrosine content falls below 20 mg./100 ml.

The excretion of l(−)-tyrosine in urine. Urines (24 hr. collections) were obtained from six normal men and six normal women, seven normal pregnant women, four cases of toxaemia of pregnancy, three cases of liver disease and one case of nephritis.

Table 2. *Excretion of l(−)-tyrosine in urine*

Source of sample	*l*(−)-Tyrosine content (mg./100 ml.)		24-hr. excretion of *l*(−)-tyrosine (mg.)		Difference between duplicate analyses (% of mean)	
	Range	Mean	Range	Mean	Range	Mean
			(*a*) Normal subjects			
6 normal men	0·9–1·9	1·4	16–39	25	2–10	5·5
6 normal women	1·0–1·7	1·3	11–28	19	2–9	4·9
7 normal pregnant women	2·2–6·2	3·2	19–82	52	2–6	5·1
			(*b*) Individual abnormal urines			
Toxaemia of pregnancy	2·7		35		2	
Toxaemia of pregnancy	2·1		17		6	
Toxaemia of pregnancy	0·9		17		8	
Cirrhosis of liver. Case 'C': 24. iv. 46	3·6		42		1	
9. v. 46	3·9		68		Single estimation	
Case 'N'	3·8		14		9	
Acute yellow atrophy of liver	115		—		1	
Chronic nephritis	1·8		22		9	

Toluene was added as preservative, and duplicate analyses carried out on the day after the completion of the collection. Loss of *l*(−)-tyrosine was found if appreciable bacterial decomposition of the urine was allowed to occur. The results of these analyses are given in Table 2. *l*(−)-Tyrosine was also found in single samples of urine from normal pigs (♂ and ♀), sheep and dog in concentrations of 0·5, 0·5, 1·6 and 0·7 mg./100 ml. respectively, but analysis of these urines presents difficulties due to deep pigmentation and precipitation of much solid matter on concentration, and the technique used for human urine is not completely satisfactory for these samples.

DISCUSSION

The chief difficulty in estimating *l*(−)-tyrosine in urine concentrates by means of the specific decarboxylase is the inhibitory effect of high concentrations of urea. A urea concentration of 6% (w/v) or less does not seriously interfere with the enzyme action, and the urea content of the concentrate can be kept down to this level by evaporating the urine in the presence of excess oxalic acid. Urea oxalate has a solubility at 20° of approximately 9·4% (w/v) in 0·2M-citrate buffer at pH 5·5. This corresponds to 5·3% (w/v) urea, which is slightly below the values generally found in concentrates. If the copious precipitate of urea oxalate and other solids is well washed with successive small volumes of cold water good recovery of *l*(−)-tyrosine is obtained (Table 1). Concentration is carried out by vacuum distillation in order to avoid excessive darkening, which would interfere with colorimetric adjustment of pH, and possible destruction of *l*(−)-tyrosine.

No difference is apparent between the *l*(−)-tyrosine concentration in the urine of normal men and normal women. The concentration is less variable than the daily output. There is increased concentration of *l*(−)-tyrosine in the urine in normal pregnancy of about 6 months' duration. It is of interest to note that the concentration of histidine is also raised in the urine during normal pregnancy (Kapeller-Adler, 1933). It may be that further work with sensitive and specific methods will show that the excretion of other amino-acids is similarly influenced by pregnancy. As was to be expected, the cases of liver disease showed increased concentration of *l*(−)-tyrosine in the urine. The increase was slight in two cases of cirrhosis of the liver without very severe symptoms, but in the case of acute yellow atrophy of the liver, which rapidly terminated fatally, a concentration of 115 mg. of *l*(−)-tyrosine/100 ml. of urine was observed. Tyrosine crystals were not found on microscopical examination of this urine.

SUMMARY

1. The determination of *l*(−)-tyrosine in urine by means of *l*(−)-tyrosine decarboxylase is described.

2. The concentration of *l*(−)-tyrosine in urine from 12 normal adults varied from 0·9 to 1·8 mg./100 ml. (mean 1·3 mg./100 ml.), and the total daily excretion varied from 11 to 37 mg. (mean 23 mg.).

3. An increased concentration of *l*(−)-tyrosine was found in urine from seven normal pregnant women and three cases of liver disease.

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A Note on the Calibration of Barcroft Differential Manometers

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Manometers used for tissue respiration experiments may be calibrated: (i) by introducing known volumes of gas from a pipette (Munzer & Neumann, 1917), (ii) by filling the apparatus with mercury, determining the volume of the several parts, and calculating the constant from the appropriate formula, (iii) by carrying out a chemical reaction in the apparatus which results in the absorption or evolution of a known amount of gas.

The number of reactions suitable for the chemical calibration of manometric apparatus is small, and, so far as the author is aware, no direct comparisons between the results of chemical and physical calibration have been made on an extensive scale. Of the reactions available for chemical calibration, the absorption of oxygen by ferrous hydroxide in alkaline solution, proposed by Harrison (1933), seems particularly well suited to the purpose, since the reagents, unlike those required for some of the methods, are in common use in the laboratory and the iron solutions may readily be standardized with permanganate or dichromate. It has the further advantage of reproducing the conditions under which the manometers are to be used, since the gas exchange measured is an uptake of oxygen.

Gibson (1943) used the method of Harrison (1933) for calibrating Barcroft manometers at 17° with good agreement between the chemical and physical methods, and when it became necessary to calibrate a number of similar manometers for use at 37° the method was again employed. It was now found that there was a systematic difference between the results given by the chemical method and the physical method of Dixon (1943). In the present paper it is shown that changes in vapour pressure on mixing the solutions used were responsible for this difference and that such changes in vapour pressure may be a source of appreciable error in accurate manometric work, for example in calibration or in the liberation of CO_2 by the addition of strong acid in an R.Q. determination.

METHODS

Apparatus. The manometers used were of the standard Barcroft type with flasks of about 35 ml. capacity. They were shaken at a rate of 120 complete oscillations/min. through a stroke of 2 in. The specific gravity of the manometric fluid was 0·8. All apparatus used was calibrated under working conditions.

Reagents. The iron solutions were prepared by dissolving 4·6–4·8 g. $FeSO_4.7H_2O$ in 50 ml. 0·2 N-H_2SO_4 and diluting to 500 ml. with water. The solutions were standardized at the time of use by titrating portions of 50 ml. with 0·1 N-$KMnO_4$. The permanganate was filtered through a sintered glass funnel and standardized against dried and powdered sodium oxalate (A.R.). The standardization of the iron and of the permanganate was repeated daily in triplicate.

Method. 2 ml. iron solution were delivered into the main chamber of the manometric flasks, using a microburette, and 0·2 ml. 2·5 N-NaOH (approx.) measured into Keilin tubes which were suspended from the centre well of the manometric flasks. The flasks were then attached to the manometers and worked firmly on to the ground joints. Thirty minutes at 37° was allowed for temperature equilibration. The taps were closed and an initial reading taken. The Keilin tubes were then displaced and the manometers shaken for a further period of 30 min. At the end of this time the final reading was taken.

RESULTS

Control values. In each experiment two manometers were set up with water in place of the iron solution. It was found that these control manometers showed an apparent absorption of some 10–12 μl. of gas on mixing the alkali with the water. It seemed that this effect might be due to lowering of the vapour pressure of the solution in the main

compartment of the manometer by the alkali. To test this, experiments were carried out in which various amounts of alkali were added to various amounts of water; in other experiments the alkali and the water were replaced by strong salt solutions. The 'absorption of gas' (Table 1) did not depend on

Table 1. *Changes in level of manometric fluid on mixing contents of Keilin tube and manometer flask*

Vol. of contents of Flask (ml.)	Vol. of contents of Keilin tube (ml.)	Change in level of manometric fluid (mm.)
Water	10 N-NaOH	
3·6	0·4	−15·2
2·7	0·3	−14·8
1·8	0·2	−16·1
0·9	0·1	−17·0
0·8	0·2	−33·9
1·9	0·1	− 6·8
1·8	0·2	−14·5
1·7	0·3	−22·7
102% (w/v) NaBr		
1·8	0·2	− 3·2
1·6	0·4	− 2·2
Water	102% (w/v) NaBr	
1·6	0·4	−13·3
1·8	0·2	− 7·3

The values in this table have been given in terms of mm. of manometric fluid. Their conversion into terms of μl. of gas by using the constants for the apparatus would be misleading, since the size of the effect does not depend on the value of the constant.

the absolute amount of NaOH used, but on the relative proportions of alkali and water, increasing as the proportion of water was diminished. The effect does not depend on any specific action of the alkali, as similar results were obtained with strong solutions of NaBr. Further, the effect is vanishingly small if NaOH is added to NaBr solution. These results agree with the suggestion that the changes in pressure observed in the control manometers are due to changes in vapour pressure of the solutions. The changes in pressure are somewhat less than the theoretical values, probably because (*a*) the addition of salts drives some gas out of physical solution, (*b*) the strong salt solutions are present in open Keilin tubes, so that the contents of the manometers are not in true equilibrium, and distillation is occurring from the main compartment to the Keilin tube. The initial level of the pressure inside the manometer is thus somewhat less than that corresponding to the true vapour pressure of the solution in the flask, and so is not decreased by the full theoretical amount on mixing the solutions in the Keilin tube and flask.

Correction of iron calibration results. A series of ten calibration experiments was carried out with each of ten manometers, and the constants compared with the values obtained by calibration with mercury (Dixon, 1943). It was found that after taking into account the lowering of vapour pressure due to the addition of the alkali the numerical values of the constants found by iron calibration were some 2% higher than those given by the mercury method. It seemed possible that the small systematic error remaining might be explained by the change of vapour pressure occurring when the iron was precipitated out of solution by the alkali at the beginning of the experiment. A rise in vapour pressure would result, and this would decrease the reading shown by the manometer for a given amount of oxygen. With the reagents used the reactions taking place were:

$$FeSO_4 + 2NaOH = Fe(OH)_2 + Na_2SO_4,$$

and $$H_2SO_4 + 2NaOH = 2H_2O + Na_2SO_4.$$

In order to calculate the change in vapour pressure resulting from these reactions, the change in the freezing-point of the solution was computed, using the values for the salts concerned at the concentrations actually present (*International Critical Tables*, 1933). The precipitation of M/30-$FeSO_4$ results in a rise in freezing-point of 0·096°, while the exchange of M/15-NaOH for M/30-Na_2SO_4 raises the freezing-point by a further 0·069°. In the neutralization of the H_2SO_4 in which the $FeSO_4$ is dissolved, 0·01 M-H_2SO_4 and 0·02 M-NaOH are exchanged for 0·01 M-Na_2SO_4: the corresponding change in freezing-point is an increase of 0·064°. Summing the changes in freezing-point, and neglecting changes in the concentration of water, the effect of which is vanishingly small, it is found that the net change in freezing-point is an increase of 0·229°. Taking the water vapour pressure at 37° as 47 mm. Hg, and assuming that the degree of ionization of the salts is substantially independent of temperature, this corresponds to a rise in vapour pressure of 0·098 mm. Hg, equivalent to 1·8 mm. manometric fluid, sp.gr. 0·8, in a capillary inclined at 15° to the vertical. The correction to be applied under other conditions of temperature, specific gravity of manometric fluid, and iron concentration may be calculated from the equation $C = (0{\cdot}027 + 2{\cdot}14\ \mathrm{Fe})\ 0{\cdot}288\ p/S \cos\theta$, where C is the correction required, in mm. manometric fluid, Fe is the concentration of iron in gram-molecules per litre, p is the tension of water vapour at the temperature of the experiment in mm. Hg, S is the specific gravity of the manometric fluid, and θ is the angle of tilt of the manometer tubes. It should be noted that this formula is only applicable so long as the concentration of H_2SO_4 used in preparing the iron solution is 0·02 N.

In order to check these calculations, measurements were made of the freezing-points of an iron solution of the composition specified (−0·144°), of 0·2N-NaOH (−0·684°), and of a solution containing both iron and NaOH (−0·606°). The depression of the freezing-point which would be expected for the solution containing both iron and NaOH is 0·684 + 0·144 = 0·828 if iron were not precipitated, while the depression actually observed was 0·606°, a difference of 0·222° which is close to the calculated value of 0·229°. Experiments were also carried out in which $Mg(OH)_2$ was precipitated by alkali from its sulphate, and the change in vapour pressure measured. $MgSO_4$ was chosen because it is a sulphate of the same type as $FeSO_4$, and with it the effects of the vapour pressure change would not be obscured by the absorption of oxygen by the hydroxide precipitated. The addition of the alkali to 2 ml. water produced a decrease in pressure of 11·8 mm., the addition of the same amount of alkali to the $MgSO_4$ solution gave an increase in pressure of 2·8 mm. manometric fluid. The net effect of the precipitation of the $MgSO_4$ was thus to convert a negative reading to a positive one and to produce a net increase in vapour pressure equivalent to 14·6 mm. manometric fluid.

It would appear to be justifiable, therefore, to correct the observed values of the constants to allow for this vapour pressure effect, and the corrected values found in the ten experiments are shown in Table 2. The residual systematic difference between the chemical and physical methods is not greater than ½%, while the values in individual determinations do not differ by more than 2% from the mean.

Table 2. *Values of manometer constants found by chemical calibration with ferrous sulphate, corrected for vapour pressure effect*

Manometer no.	Chemical calibration		Physical calibration	Difference (%)
	Mean	S.D.		
1	2·92	0·04	2·88	+1·3
2	3·09	0·04	3·14	−1·6
3	3·05	0·04	3·06	−0·3
4	3·24	0·03	3·26	−0·6
5	2·63	0·04	2·62	+0·3
6	2·86	0·03	2·90	−1·3
7	2·98	0·05	2·99	−0·3
8	2·81	0·02	2·84	−1·0
9	2·88	0·03	2·89	−0·3
10	2·77	0·02	2·78	−0·3
Averages	2·923	—	2·936	−0·4

The values in the column 'Chemical calibration' are constants for oxygen at 37°, fluid volume 2·2 ml., oxygen uptakes of about 400 μl. Each figure is the average of 10 determinations. The figures in the column 'Physical calibration' are the constants calculated from the formula of Dixon (1943).

The following figures illustrate the method of applying the corrections. At 30 min. the manometric readings were: control manometer, −3·3 mm.; manometer no. 9, −136·8 mm. The net manometer reading was thus 133·5 mm. To this is added 1·8 mm., the correction for the increase of vapour pressure due to Fe precipitation and neutralization of acid, giving 135·3 mm. 2·0 ml. of 0·0347N-$FeSO_4$ was used, corresponding to an oxygen uptake of 389 μl. The constant of the manometer is 389 ÷ 135·3 = 2·87.

The results showing the influence of vapour pressure changes on the manometric readings suggest that this factor may occasionally be of appreciable importance in accurate work, and should be taken into account whenever strong solutions of reagents are added to a manometric flask in the course of an experiment. The effects will, however, be much reduced at room temperature, which probably explains why Gibson (1943) found agreement between the ferrous hydroxide and physical methods.

With regard to the relative merits of the two methods of calibration, while there can be no doubt that the formula of Dixon (1943) gives highly satisfactory results, the chemical method has the advantage of testing the manometers under actual working conditions. If the physical method alone is used, defects, e.g. a badly ground tap, may not be suspected until considerable time and effort have been wasted. Moreover, with the chemical method, the constants may be checked at any time without dismantling and drying the whole apparatus.

SUMMARY

1. Under certain circumstances, vapour pressure changes may interfere with accurate measurements in micro-respirometers.

2. A method for the chemical calibration of manometers is described, depending on the absorption of oxygen by ferrous iron in alkaline solution, a reaction proposed by Harrison (1933).

3. The chemical method has been found to agree with the results of calibration by the physical method of Dixon (1943).

The author wishes to thank Professor D. C. Harrison for his interest in the work.

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British Anti-lewisite

4. ANTIDOTAL EFFECTS AGAINST THERAPEUTIC ARSENICALS

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Since the original objective of this research was the provision of an antidote for arsenical vesicants, the earlier work (Stocken & Thompson, 1941) with 2:3-dimercaptopropanol (British Anti-lewisite or 'BAL') was carried out with arsenoxides derived from chemical warfare agents, or with sodium arsenite.

After demonstrating the effectiveness of BAL in the local treatment of skin contaminated with lewisite, it was shown that systemic intoxication arising from heavy contamination with lewisite could also be satisfactorily treated in laboratory animals. It was therefore obviously desirable to determine whether BAL was also effective against the therapeutic arsenicals, with a view ultimately to the treatment of patients suffering from the toxic manifestations that sometimes occur during the course of arsenotherapy. About the same time that this experimental work was commenced a start was made in the collection and study of a series of patients showing toxic reactions developing during treatment with therapeutic arsenicals. The results of this clinical study are reported elsewhere (Carleton, Peters, Stocken, Thompson, Williams, Storey, Levvy & Chance, 1946). The only previous work in man in which lesions other than purely experimental contaminations due to chemical warfare agents had been studied was that of Longcope, Wintrobe & Luetscher (1943) on the use of BAL in the treatment of arsenical dermatitis occurring in factory operatives. The problem of the therapeutic arsenicals was simultaneously begun in this Department and in the U.S.A. by Eagle, who, in 1943, reported on the effectiveness of BAL in resuscitating trypanosomes from the effects of mapharside, and succeeded in saving rabbits by the injection, 5 min. after lethal doses of mapharside, of BAL dissolved in propylene glycol.

The work described here deals with four arsenicals, representative of the various chemical types used in therapy—mapharside, arsphenamine, neoarsphenamine and a pentavalent arsonic acid—and has already been reported to the Ministry of Supply (Stocken, Thompson & Whittaker, 1944; Whittaker, 1945). Earlier work on the inhibitory action of therapeutic and other arsenicals on the respiratory enzyme system of cells has already been reviewed (Peters, Sinclair & Thompson, 1946).

Two types of experimental evidence have been obtained which demonstrate the effectiveness of BAL against these therapeutic arsenicals: (*a*) experiments to demonstrate the protection and reactivation of the brain pyruvate oxidase system from their inhibitory action. (*b*) A study of the survival rate of rats treated with BAL after poisoning by these arsenicals.

EXPERIMENTAL

Arsenicals. Freshly prepared solutions of the following arsenicals were used:

Mapharside (3-amino-4-hydroxyphenylarsenoxide hydrochloride): samples of 'undiluted' mapharside were kindly provided by Parke, Davis and Co.

Arsphenamine (3:3′-diamino-4:4′-dihydroxyarsenobenzene hydrochloride): this was prepared from 3-amino-4-hydroxyphenylarsonic acid (cf. Raiziss & Gavron, 1923).

Neoarsphenamine (sodium 3:3′-diamino-4:4′-dihydroxyarsenobenzene-*N*-methylenesulphoxylate): the commercial product of May and Baker Ltd. The dry solid arsenical was kept in a nitrogen-filled desiccator, and no increase in toxicity was observed over an interval of many months.

3-Amino-4-hydroxyphenylarsonic acid: kindly supplied by May and Baker Ltd.

Thiols. The sample of BAL used throughout was from a water-purified batch described by Peters, Stocken & Thompson (1944). Its thiol content was 99–100% of the theoretical iodine titration. 2-Mercaptoethanol was a commercial preparation and had a thiol content of 87% theoretical (iodine titration); this was allowed for in the enzyme experiments.

Enzyme experiments. Two types of pyruvate oxidase preparation have been used, activity being determined by the Warburg technique in each case.

(1) Rat brain *brei*. The minced brain (Passmore, Peters & Sinclair, 1933) was added to Ringer-phosphate solution (pH 7·3) in tared Warburg bottles containing sodium pyruvate in a final concentration of 0·02 M. Respiration was measured in air for at least 1 hr. at 38°.

(2) Pigeon brain dispersion, prepared by the method of Banga, Ochoa & Peters (1939). The brain, pounded to a homogeneous cream in an ice-cold mortar, was ground with ice-cold 0·9% KCl. The dispersion was then pressed through muslin, 1·3 ml. 0·5 M-phosphate buffer (pH 7·3) added, and the volume adjusted to 16·5 ml. with more 0·9% KCl. 2 ml.

of this mixture were added to each of 8 bottles, standing in ice, containing 0·006 M-pyruvate, 0·004 M-fumarate and appropriate amounts of thiol and arsenical, and enough water to a final volume of 3 ml. Measurements, again in air, were usually carried out for 30 min., oxygen consumption being expressed as μl. O_2/2·0 ml. dispersion/30 min. Determinations were made in duplicate with both types of preparation.

The percentage inhibitions due to the arsenical alone, or in the presence of thiol, or, in reactivation experiments, the percentage reactivation of enzyme by thiol, are given by the following expressions:

If x_0 = mean rate of O_2 consumption in control bottles,
x = mean rate in bottles containing thiol,
y = mean rate in bottles containing arsenical,
z = mean rate in bottles containing arsenical + thiol,

$$\text{Inhibition due to arsenical } (I_0) = 100\,\frac{(x_0 - y)}{x_0}\,\%,$$

$$\text{Inhibition due to arsenical + thiol } (I) = 100\,\frac{(x - z)}{x}\,\%,$$

$$\text{Percentage reactivation of enzyme} = 100\,\frac{(I_0 - I)}{I_0} = 100\,\frac{(x_0 z - xy)}{x\,(x_0 - y)}.$$

RESULTS

Enzyme experiments

Mapharside. Using the rat brain *brei*, a final concentration of 0·07 mM-mapharside caused under our conditions approximately 50 % inhibition of the total respiration in the presence of pyruvate. In the protection experiments, the BAL, freshly dissolved in Ringer-phosphate, was added to the brain sample dispersed in the Ringer-pyruvate mixture, the mapharside being added 2–3 min. later.

Table 1 shows the mean percentage inhibition of pyruvate oxidation brought about by mapharside in the absence of and in the presence of 1·7 and 3·4 equiv. of BAL (1 mol. BAL ≡ 1 mol. mapharside). Three experiments are also included to show that 1·8 and 3·6 equiv. of 2-mercaptoethanol confer no protection; in each case the inhibition was actually increased by the monothiol, though the interpretation of this is not clear.

Table 1. *Protection of rat brain* brei *pyruvate oxidase system from inhibition by mapharside*

(Conc. mapharside 0·07 mM.)

Exp.	Thiol	Thiol. concentration (mM.)	Inhibition over 1 hr. (%) Mapharside	Thiol + Mapharside
1	BAL	0·12	50	17
2	,,	0·12	59	34
3	,,	0·12	55	21
1	,,	0·24	50	2
4	,,	0·24	56	0
5	,,	0·24	37	0
6	,,	0·24	59	0
7	,,	0·24	62	13
6	2-Mercaptoethanol	0·26	59	66
2	,,	0·51	59	78
7	,,	0·51	62	80

A similar result was obtained using the brain dispersion (Table 2); a considerable measure of protection was again provided by 1·5 equiv. of BAL, 3 equiv. affording complete or almost complete protection. It will be noted from Table 2 that the addition of BAL causes a considerable increase in the oxygen uptake of the dispersion. This effect, which is not obtained with the brain *brei*, is further discussed in an Appendix to this paper.

Table 2. *Effect of BAL on the mapharside* ('*Maph.*') *inhibition of pyruvate oxidation by brain dispersions*

(Conc. mapharside 0·014 mM.)

Equiv. of BAL	O_2 uptake (μl. O_2/30 min./2 ml. dispersion) Control	Maph.	BAL	BAL + Maph.	Inhibition (%) Maph.	Maph. + BAL
1·5	333	216	459	416	35	9
1·5	288	142	336	301	52	10
1·5	314	148	407	336	53	17
3·0	335	144	272	277	57	0
3·0	437	232	512	495	47	3
3·0	327	154	444	424	53	5

A few experiments have been carried out with the brain *brei* to determine whether the addition of BAL to the system, already poisoned with mapharside, can bring about any reversal of the inhibition of enzyme activity.

In these experiments (Table 3) the BAL was placed in the side-bulbs, and the mapharside added to the unprotected brain sample in the main compartment of the flask. The manometers were then attached to the thermostat, and 15 min. allowed for temperature equilibration at 38°, after which the side-bulb contents were tipped into the main compartment, and the inhibition measured as before over two succeeding half-hour periods. The total period of poisoning before the addition of the thiol was 20 min., of which the last 15 min. were spent in the thermostat at 38°. In each experiment, bottles with no added thiol were included, so that a direct comparison could be obtained between the degree of inhibition in the completely unprotected system and that remaining in the bottles to which a thiol was added after 20 min. poisoning.

Although in the experiments summarized in Table 3 no measurements were made of the mapharside inhibition before the addition of BAL, the experiments, in our opinion, do provide true evidence of reversal, in that our earlier work has indicated that inhibition of this system by mapharside (or arsenite or lewisite oxide) is rapid, and remains constant over three or four succeeding 15 min. periods.

Table 3. *Reactivation by BAL of rat brain* brei *pyruvate oxidase system after poisoning by* 0·07 mM-*mapharside*

Exp. no.	Inhibition over 1 hr. (%) Maph. alone	Maph. + BAL	Reactivation of enzyme (%)
	0·24 mM-BAL		
1	58	21	64
2	58	21	64
3	54	17	69
4	53	16	70
Mean	56	19	67
	0·48 mM-BAL		
1	58	26	55
2	58	8	86
3	54	7	87
4	53	16	70
Mean	56	19	72

this time BAL was tipped into four of the bottles (two controls and two with neoarsphenamine), and the measurements of oxygen uptake resumed in all bottles from 10 to 30 min. Since the brain dispersion had been added 15 min. before zero reading (20 min. in the case of the first exp.) the system had been poisoned for a total of about 20 min. before the addition of the thiol. Under these conditions BAL is able to effect a mean reactivation of the poisoned enzyme of about 60 % in the course of the first 20 min. after its addition to the system.

Arsphenamine. This arsenical was found to have very nearly the same toxicity towards pyruvate oxidation in either brain *brei* or dispersion as has neoarsphenamine. Table 7 shows that BAL is also effective in detoxicating this arsenical, whereas a concentration of 2-mercaptoethanol equivalent to twice that of BAL is almost completely inactive.

Table 4. *Relative sensitivities of pigeon brain* brei *and dispersion to therapeutic arsenicals*

Arsenical	*Brei* (0·02 M-pyruvate) No. of exp.	Concentration of arsenical (mM)	Mean inhibition (%)	Dispersion (0·006 M-pyruvate) No. of exp.	Concentration of arsenical (mM)	Mean inhibition (%)	Ratio of As concentrations
Mapharside	7	0·07	61	8	0·014	53	5
Neoarsphenamine	7	0·22	61	7	0·07	72	3
Arsphenamine	4	0·21	50	6	0·07	49	3

Neoarsphenamine. Preliminary experiments with the rat-brain *brei* showed that BAL was also able to protect this system from inhibition by neoarsphenamine, 2-mercaptoethanol being again, under our conditions, ineffective. Relatively high concentrations (of the order of 0·2 mM) of neoarsphenamine were, however, required to produce 50 % inhibition of oxygen uptake, and a concentration of BAL equal to or greater than this produced by itself a variable but marked effect on the respiration, often increasing the oxygen uptake slightly during the first 30 min., and inhibiting it to the extent of about 20 % during the second 30 min. period. For these reasons the pigeon-brain dispersion was investigated as an alternative test system, since it was known from earlier work (Sinclair & Thompson, 1940) that dispersions are more sensitive to arsenicals than the brain *brei*. A comparison of the relative sensitivities of the pigeon-brain *brei* and dispersion to three types of therapeutic arsenical is given in Table 4.

A comparison of the relative effectiveness of BAL and 2-mercaptoethanol in protecting the pyruvate oxidation system present in brain dispersion from the inhibitory effect of neoarsphenamine is given in Table 5.

Table 6 shows the results of reactivation experiments with BAL. In these experiments the inhibition produced by the neoarsphenamine was measured for the first 5 min. in all the bottles. At the end of

Table 5. *The effect of BAL and 2-mercaptoethanol on the inhibition by neoarsphenamine ('Neo.') of pyruvate oxidation in pigeon brain dispersions*

(Concentration of neoarsphenamine 0·07 mM.)

Ratio SH:As	O_2 uptake (μl. O_2/30 min./2 ml. dispersion) Control	Neo.	Thiol	Thiol + Neo.	Inhibition (%) Neo.	Neo. + thiol
			BAL			
6	301	55	460	477	82	0
6	314	91	501	517	71	0
7	410	159	534	534	61	0
			2-Mercaptoethanol			
12	309	89	336	149	71	56
12	243	73	305	91	70	70
12	263	70	255	93	73	64

Table 6. *The reactivation of pyruvate oxidation in brain dispersions by BAL after poisoning with* 0·07 mM-*neoarsphenamine*

Ratio SH : As	Inhibition (%) Neo. (0–5 min.)	Neo. (10–30 min.)	Neo. + BAL (10–30 min.)	Reactivation by BAL (%)
6	69	76	33	58
6	46	56	30	45
6	56	70	21	70
12	55	75	35	53
12	66	80	18	77
12	64	80	21	74

The reactivation experiments also given in Table 7 were carried out exactly as for those already described for neoarsphenamine.

Table 7. *Effect of BAL on the arsphenamine inhibition of pyruvate oxidation by brain dispersions*

(Concentration arsphenamine 0·07 mM.)

A. Protection experiments

Ratio SH:As	O_2 uptake (μl. O_2/30 min./2 ml. dispersion) Control	Arsph.	Thiol BAL	Thiol + arsph.	Inhibition (%) Arsph.	Arsph. + thiol
6	329	171	493	481	48	2
6	344	245	500	509	29	0
6	269	111	443	436	59	2
			2-Mercaptoethanol			
12	349	189	472	281	46	40
12	347	176	493	251	49	49
12	479	235	531	316	51	40

B. Reactivation experiments

Ratio SH:As	Inhibition (%) Arsph. (0–5 min.)	Arsph. (10–30 min.)	Arsph. + BAL (10–30 min.)	Reactivation by BAL (%)
12	38	61	13	79
12	32	43	14	68
12	40	48	19	60

3-Amino-4-hydroxyphenylarsonic acid. From the work of Voegtlin & Smith (1920) and others, it has been realized for many years that pentavalent arsenicals are markedly less toxic than the corresponding trivalent compounds. A few experiments, however, were carried out to test the toxicity of 3-amino-4-hydroxyphenylarsonic acid to the brain pyruvate oxidase system, with a view ultimately to investigating the protection of the system by BAL if any significant inhibition should have been produced by the arsenical at suitable concentrations.

Even with relatively large concentrations of the arsonic acid, however, only small and insignificant inhibition of enzymic activity was produced (Table 8).

Table 8. *Effect of 3-amino-4-hydroxyphenylarsonic acid on the brain pyruvate oxidase system*

Concentration of arsenical (mM)	Oxygen uptake* Control	Arsenical	Inhibition (%)
	Brei		
0·3	1515	1529	0
0·6	1515	1542	0
0·9	1515	1497	1
1·2	1515	1502	1
1·4	1515	1324	12
	Dispersion		
0·3	420	401	5
1·4	420	352	16

* For *brei*, expressed as μl. O_2/hr./g.; for dispersion, μl. O_2/30 min./2 ml.

Animal experiments

The ability of injected BAL to cause the survival of rats after injection of lethal amounts of mapharside or neoarsphenamine has also been demonstrated.

Mapharside. White rats, 150–200 g. each, were used. Mapharside, freshly dissolved in glass-distilled water, was injected intramuscularly into the hind-leg, and at intervals varying from 5–15 min. after injection a solution of BAL in propylene glycol was injected intramuscularly in doses of 40 mg./kg. body weight into the opposite hind-leg. The results of these experiments are given in Table 9. It will be seen that complete protection is afforded by a single dose of BAL given 5–6 min. after the arsenical. The amount of BAL required to achieve this protection is only of the order of 2 equiv. When the same dose of BAL was injected 15 min. after giving the arsenical 66% of the animals survived; it should be pointed out, however, that 15 min. after receiving this dose of mapharside intramuscularly the rats, under our conditions, already show marked signs of generalized intoxication, many being in a state of collapse.

Table 9. *Effect of injected BAL (40 mg./kg.) on the survival of rats after injection of lethal doses of mapharside*

Exp.	No. rats used	Mapharside (mg./kg. body wt.)	Interval before giving BAL (min.)	Survivors No.	Survivors %
		Control			
1	4	18–19	—	1	25
2	14	28–32	—	3	21
3	4	43–44	—	0	0
4*	6	45–52	—	1	17
5	6	42–45	—	0	0
		Treated			
2	6	30–33	15	4	66
2	6	30–32	7	6	100
4*	5	37–40	7	5	100
5	6	43–47	5	6	100

* 3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, prepared in this laboratory, used instead of mapharside; dose expressed as mg. mapharside.

Neoarsphenamine. Preliminary experiments with neoarsphenamine showed that, unlike mapharside, the onset of poisoning is slow. Doses of about 200 mg./kg. cause about 70% fatalities in 3–6 days, whereas doses of about 170 mg./kg. cause fatalities over an even longer period. This would be in accordance with the view put forward by Voegtlin & Smith (1920) that arsenobenzene and pentavalent arsenical compounds exert their toxic effect only after conversion to a trivalent arsenoxide form. Owing to the more chronic type of poisoning produced by neoarsphenamine the injections of BAL were repeated for three successive days.

White rats of about 200 g. weight were injected with an aqueous solution of neoarsphenamine intramuscularly into a hind-leg, the dose being contained in 0·2 ml. of solution. In animals receiving BAL the dose of arsenical was followed 15 min. later by one of BAL of approx. 70 mg./kg. in 0·15 ml. propylene glycol injected intramuscularly into the other hind-leg, and repeated every 24 hr. for three successive days. The animals were kept under observation for 1 month. The results are shown in Table 10.

Table 10. *Effect of BAL on the survival of rats after lethal doses of neoarsphenamine*

Exp.	Dose of neoarsph. (mg./kg.)	Dose of BAL (mg./kg.)	No. of rats used	Deaths: No.	Deaths: Time of survival (days)	Survivors: No.	Survivors: %
			Control				
1	126–134	—	4	—	—	4	100
2	161–166	—	4	2	4	1	25
				1	9		
3	175–188	—	4	2	3	1	25
				1	5		
4	188–214	—	10	4	4	3	30
				2	5		
				1	6		
			Treated				
3	184–194	77–81	3	—	—	3	100
4	182–212	66–80	10	—	—	10	100

DISCUSSION

The results described here with the pyruvate oxidase system of rat and pigeon brain show that good protection against the inhibitory action of three therapeutic arsenicals, mapharside, neoarsphenamine and arsphenamine, can be obtained by the addition to the system of small amounts of BAL. In the case of mapharside almost complete protection is afforded by the presence of 3 equiv. of BAL. With neoarsphenamine 6 mol. of BAL/mol. were effective. With neither type of arsenical was the one monothiol investigated (2-mercaptoethanol) effective, although 24 mol. were used in the case of neoarsphenamine. In this connexion it should be noted that a high relative concentration of glutathione (10 : 1) was required by Voegtlin, Rosenthal & Johnson (1931) to prevent the reduction in total respiration of animal tissue by arsenoxide; when sodium arsenite was used instead of arsenoxides, 40 mol. of glutathione were required, a ratio of 10 : 1 being entirely without effect.

Experiments with rats following the injection of lethal amounts of these arsenicals has shown that BAL can act as an effective detoxicant. It was concluded from this work that a strong case existed for the testing of BAL as a therapeutic agent in man against the toxic effects of arseno-therapy.

Considering the narrower point of the relative toxicities of the arsenicals used here it is interesting to note that the results obtained with the brain pyruvate oxidase system are in general agreement with those described by Voegtlin and his colleagues for other tissues, thus supporting the view that arsenicals exert their toxic effect through conversion to an 'active' arsenoxide form.

SUMMARY

1. In extension of earlier work with sodium arsenite and the chemical warfare arsenicals, British Anti-Lewisite (BAL) has been tested as an antidote to the toxic action of mapharside, neoarsphenamine and arsphenamine.

2. Using the pyruvate oxidase system of brain it has been shown that BAL can protect effectively against these three arsenicals, and can also bring about a significant degree of reactivation of the already poisoned enzyme system.

3. BAL is also effective in bringing about the survival of rats after the injection of lethal doses of mapharside or neoarsphenamine.

This work was carried out for the Ministry of Supply as part of a programme of research under the direction of Prof. R. A. Peters, M.C., F.R.S. Our thanks are due to the Chief Scientific Officer, Ministry of Supply, for permission to publish, and to Miss M. R. Kempson for technical assistance.

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Appendix. The Effect of BAL on Pyruvate Oxidation in Pigeon Brain Dispersions

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It will be noted in Tables 2, 5 and 7 of the preceding paper that BAL and to a lesser extent 2-mercaptoethanol cause a considerable increase in the oxygen uptake of pigeon brain dispersions in the presence of pyruvate. A similar effect was observed by Peters & Wakelin (1941) with kerateine. It was obtained so consistently with all concentrations of BAL used in these experiments that it was thought worth while to find out whether it was actually due to an increased oxidation of pyruvate.

In Table 1, the measured difference in the pyruvate concentrations at the end of the experiment between bottles containing pyruvate alone and pyruvate with BAL is compared with the difference in pyruvate concentration calculated by means of McGowan's quotient (McGowan, 1937; 1 mg. pyruvate = 450 μl. O_2) from the increase in oxygen uptake in the BAL bottles over the controls.

Brain dispersions were prepared as described in the previous paper. At the end of each experiment (duration 40 min.), protein was precipitated with 2 ml. 25% (w/v) trichloroacetic acid and the bisulphite-binding capacity (B.B.C.) estimated by the method of Clift & Cook (1932). The difference in B.B.C. is expressed as mg. pyruvate (1 ml. N/200 iodine ≡ 0·275 mg. pyruvate).

Table 1. *The effect of BAL on the pyruvate oxidation of pigeon brain dispersions*

(*a*) = 2 ml. dispersion + 0·006 M-pyruvate + 0·004 M-fumarate.
(*b*) = (*a*) + 0·5 mM-BAL.

(Numbers in brackets are number of bottles averaged.)

O_2 uptake (μl.) in 40 min.		Increase in O_2 uptake (*b*) − (*a*) due to BAL		Difference (*a*) − (*b*) in bisulphite-binding capacity after 40 min. (mg. pyruvate)
(*a*)	(*b*)	μl. O_2	mg. pyruvate	
320(2)	510(1)	190	0·42	0·44
276(2)	333(1)	57	0·13	0·18
332(3)	530(4)	198	0·44	0·57

In each case the B.B.C. of the bottles containing BAL was lower than that of the controls, the difference running parallel with the extra oxygen uptake in the BAL bottles. In the second experiment, the effect of added BAL was less than usual on this occasion (the BAL solution having stood for over half an hour before addition of the dispersion); significantly, the difference in bisulphite binding is less marked also.

Table 2 shows that the increased oxygen uptake is not due to the oxygen uptake of BAL alone, resulting from oxidation of the BAL during incubation at 38° in air, or to an effect of BAL on the residual respiration (in presence of 0·004 M-fumarate but no pyruvate), since both are small and of the same order of magnitude.

Table 2. *The effect of 0·5 mM-BAL on the residual respiration of pigeon brain dispersions*

(O_2 uptake expressed as μl./40 min./2 ml. dispersion.)

Dispersion	Dispersion + BAL	Increase due to BAL	BAL alone
153	177	24	21
141	163	22	3

The conclusion drawn from these experiments, that BAL stimulates the oxidation of pyruvate by pigeon brain dispersions, is not unexpected. Thiol groups are believed to be essential to the activity of one or more of the protein components of the pyruvate oxidase system (cf. Peters & Wakelin, 1946); the addition of thiol would be expected to increase the number of these, thereby increasing also the enzymic activity of the whole system.

SUMMARY

The increase in oxygen uptake by pigeon brain dispersion in the presence of pyruvate brought about by low concentrations of BAL has been shown to be due to a stimulation of the enzymic activity of the pyruvate oxidase system.

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Preparation and Pharmacological Properties of 4-Hydroxymethyl-2-(3′-amino-4′-hydroxyphenyl)-1:3-dithia-2-arsa*cyclo*pentane (Mapharside-BAL Compound)

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As an extension to the work on the provision of an antidote to the arsenical war gases it has been shown in the United Kingdom (Stocken & Thompson, 1941; Stocken, Thompson & Whittaker, 1944) and in the United States (Eagle, 1943) that injections of 2:3-dimercaptopropanol (BAL) will save animals poisoned with lethal amounts of mapharside, if injected within 7 min. There is also evidence that BAL has a marked therapeutic effect in patients suffering from the toxic complications of arsenical therapy (Longcope, Wintrobe & Luetscher, 1943; Carleton, Peters, Stocken, Thompson & Williams, 1944). Nevertheless, it was thought advisable to investigate directly the toxicity of the compound of BAL and mapharside, which has been prepared in this laboratory and will be here called Maph-BAL. Previous experiments have shown clearly that the lewisite-thioarsinite has a toxicity of less than one-fifth that of the parent compound. On the other hand, to our surprise, Maph-BAL under certain conditions is more toxic than mapharside; in agreement with the lewisite experiments, however, its toxicity is diminished to one-fifth that of mapharside in the presence of extra BAL.

EXPERIMENTAL

Preparation of mapharside-BAL

Maph-BAL was prepared by dissolving 1 mol. of mapharside and 1 mol. BAL in ethanol (20 times the vol. of BAL) and evaporating the solution to crystallization in a vacuum desiccator. The first crop of crystals was 70% of the theoretical, m.p. 172–3° (uncorr.) The mother liquor was rejected since precipitation by ether gave a sticky, coloured product. (Found: As, 22·7%; S, 18·9%; Cl, 10·3%. $C_9H_{13}O_2NClS_2As$ requires: As, 22·76%; S, 18·75%; Cl, 10·4%.) On standing, the compound remained unchanged for several weeks when kept in the dark, but after 2 years became discoloured.

Injection experiments

In order to obtain a comparison of the toxicities of mapharside with Maph-BAL, groups of rats were injected intramuscularly in the hind leg with solutions of the compounds in a 1:2 mixture of water and propylene glycol. The solutions were made up immediately before use. Table 1 gives the results obtained under comparable conditions and other additional results obtained either as controls for later experiments or at times when the two substances were not injected on the same day into rats of the same weight. Throughout this paper the dosage level of Maph-BAL given in the tables is calculated from the mapharside content; to obtain the absolute amount of Maph-BAL injected the figures must be multiplied by the factor 1·45.

From Table 1 it will be seen that whereas the LD_{50} dose for mapharside is approximately

Table 1. *Direct comparison of toxicity for mapharside* (*Maph*) *and mapharside-BAL* (*Maph-BAL*)

(*a*) Experiments done by comparison at same time and with rats of same weight

		Maph			Maph-BAL		
			Survivors			Survivors	
Date	Dose (mg./kg.)	No. of animals	No.	%	No. of animals	No.	%
24. xi. 44	20	20	7	35	20	0	0
11. xii. 44	15	10	5	50	10	0	0
29. i. 45	10	10	10	100	10	0	0
29. i. 45	7	10	10	100	10	0	0
(*b*) Other experiments							
6. xi. 44	40	6	0	0	—	—	—
9. xi. 44	30	20	3	15	—	—	—
Dec., Jan. and Feb.	14–15	—	—	—	20	2	10
Dec. and Jan.	10	10	8	80	6	2	33
1. ii. 45	4	—	—	—	10	4	40
1. ii. 45	2	—	—	—	10	10	100

15 mg./kg., that for Maph-BAL may be as low as 4 mg./kg. The animals were kept under observation for at least 1 month.

It was observed that the rats died very much more quickly from the effects of the thioarsinite than from the mapharside, seldom in less than 6 hr. after injection of the mapharside, but were usually dead in 2 hr. after the Maph-BAL. Only one case of a late death (3rd day after injection) took place and this was with the 4 mg./kg. dosage of Maph-BAL.

Although these results show an increased toxicity due to combination of the mapharside with BAL, further experiments proved that when an excess of BAL was present, the animals were able to tolerate several lethal doses of the Maph-BAL compound. Different amounts of Maph-BAL were injected intramuscularly into one hind leg and 10 min. later 5% (w/v) BAL in benzyl benzoate-arachis oil mixture (Eagle, 1943) into the other leg. The results obtained are presented in Table 2.

Table 2. *Protective effect of BAL against the toxicity of Maph-BAL in rats*

No. of rats	Dosage of Maph-BAL (mg./kg.)	Dosage of BAL (mg./kg.)	Survivors No.	Survivors %
5	20	50–70	5	100
5	40–45	„	5	100
10	63–71	„	8	80
11	90–95	„	7	64
10	108–113	„	3	30
5	126–138	„	0	0

Having established that an excess of BAL could suppress the toxicity of several lethal doses of Maph-BAL, experiments were carried out to discover the minimum amount of BAL required to abolish the effects of 1 LD_{50} dose of Maph-BAL. These tests were done in the same way as described above and as will be seen from Table 3 one equivalent of BAL is adequate for complete protection.

Half an hour after injection of the Maph-BAL the animals showed signs of arsenical intoxication, many being in a state of collapse with diarrhoea and lachrymation. We have obtained some indication of the limiting time which could be allowed to elapse before administration of the antidote to ensure survival of the animal. It will be noticed from Table 4 that increasing the dose at 30 min. diminishes the mortality rate but that after longer intervals there is no effect.

Table 3. *The protection of rats against the toxic effect of Maph-BAL by minimal amounts of BAL*

No. of rats	Dosage of Maph-BAL (mg./kg.)	Dosage of BAL injected 10 min. after the Maph-BAL (mg./kg.)	No. equivalents BAL	Survivors No.	Survivors %
5	15	22	2·8	5	100
5	16	12	1·4	5	100
10	15–21	10	0·9–1·25	10	100
10	14	6	0·8	9	90
7	15–16	4	0·5	0	0

Table 4. *The effect of the time interval elapsing before injection of BAL on the survival rate of rats given Maph-BAL and BAL*

No. of rats	Dosage of Maph-BAL (mg./kg.)	Time interval (min.)	Dosage of BAL (mg./kg.)	Survivors No.	Survivors %
14	15–20	30	10	5	35
9	15–20	60	8–10	5	55
7*	15–20	90	8–10	2	28
10	15	30	50	10	100
5	61–64	30	52	5	100
10	15	45	40–43	4	40
9	15	60	60–65	2	22

* In this group more than seven had been injected but some had died before the BAL was due to be given.

Since it was found that a single dose of 4 mg./kg. BAL was inadequate to protect animals against a lethal dose of Maph-BAL, we tried the effect of repeated doses. Five rats were injected with 15 mg./kg. of Maph-BAL and 4 mg./kg. BAL, given 10, 130, 250 and 370 min. afterwards; all the animals survived. When, however, the amount of BAL was decreased to 2 mg./kg. and given under the same conditions no protection was obtained. Doses of 4 mg./kg. BAL at intervals of 4 hr. have been tolerated by men (Eagle, 1944; Sulzberger, Baer, Kanof & Eagle, 1944).

Table 5. *A comparison of the therapeutic efficiency against the toxicity of Maph-BAL of the injection of two monothiols and of BAL*

Compound	Dosage of Maph-BAL (mg./kg.)	Dose of thiol (mg./kg.)	No equivalents thiol	No. of rats	Survivors No.	Survivors %
Untreated	15	—	—	6	1	17
Glutathione	15	75	1·9	6	1	17
Cysteine ester HCl	15	46·5	1·95	6	0	0
*Cysteine ester HCl	15	475	20	6	4	66
BAL	15	10	1·25	5	5	100

* Given by intraperitoneal injection.

Glutathione (GSH) and cysteine ethyl ester HCl were also given to see whether a monothiol could suppress the toxicity of Maph-BAL. Some effect was shown with 40 mol. of the cysteine ester but neither glutathione nor the cysteine ester approaches BAL in therapeutic value. The results are shown in Table 5.

Experiments upon Protozoa

To obtain some information about the protoplasmic toxicity experiments were carried out with the ciliate *Colpidium colpoda* which has been under continuous subculture in this laboratory for many years (Peters, 1921, 1929; Walker, 1928). The technique adopted in these toxicity experiments was rather different from that described by Walker, owing to the relative non-toxicity of the mapharside. Observations were made at room temperature over periods up to 48 hr., and the toxicity was judged from the amount of the arsenical required to kill in this period. This method is not so accurate as that of rapid observation, but the differences in toxicity were large. All solutions were brought to *c.* pH 7·4 before use. Mapharside was toxic at a concentration of M/1000–M/1500; Maph-BAL was much more toxic namely at M/24,000; BAL alone was not toxic at concentrations of M/6400 or M/12,800; but at these concentrations it did not decrease or increase the toxicity of Maph-BAL. The interpretation of these observations is not clear; possibly the whole Maph-BAL molecule is toxic to the ciliate, and the fact that the temperature of this experiment was *c.* 20° lower than that of the animal experiments is consistent with this; but it may also be that under these conditions the time of observation is so long that the BAL in the dilute solutions has time to oxidize. It is hoped to elucidate these points in further work.

DISCUSSION

The difference in the independent toxicities of lewisite-BAL and Maph-BAL is indeed remarkable; such facts make generalization the despair of biological workers. Close examination of the two compounds under the same conditions of pH (pH 9–9·5) gives evidence of a slight nitroprusside reaction for the mapharside compound and none for the lewisite compound; this would indicate a slight dissociation of the former under conditions in which the lewisite compound remains intact.

A proved explanation of the toxicity of Maph-BAL cannot yet be given; nevertheless, the fact that reversal is possible when the animal is showing all the usual signs of arsenical intoxication makes it fairly certain that the toxicity of the compound is due to dissociation. This is supported by the nitroprusside experiment mentioned; further, if toxicity in the whole animal was due to the undissociated molecule, it should be increased (not decreased) by the presence of extra BAL which would tend to decrease the dissociation. It is probable that the more rapid toxic effects of the compound are due to a penetration to the sites which are dangerous to the animals, by virtue of its physical properties which are different from those of mapharside. In this way the arsenic would be liberated locally where it could exert its maximum toxic effect; the increased toxicity would then be explained as due to decreased chance of elimination, as compared with mapharside, before toxic effects ensued. It is to be noted that mapharside itself has high water solubility, which will encourage elimination from the system.

From work reported with the pyruvate oxidase system (Stocken, Thompson & Whittaker, 1944) 1 mol. BAL in excess was required to reverse the toxicity of mapharside; it is interesting to note that similarly one extra mol. is required to protect the whole animal in the case of the rat. As regards the therapeutic application of BAL in man against poisoning by substances forming arsenoxides *in vivo*, there is now a strong argument for giving the maximum amounts of BAL which can be tolerated, because excess of BAL protects against any toxicity of Maph-BAL as well as of mapharside. Though caution in interpretation is necessary, there is no present reason for thinking that any Maph-BAL reaching the circulation by liberation of As in the tissues will not have with it sufficient uncombined BAL to prevent the development of any toxic action.

SUMMARY

1. Toxicity experiments have been carried out in rats with the compound mapharside-BAL, mapharside, and mixtures of Maph-BAL and BAL.

2. In presence of 1 mol. excess BAL, Maph-BAL has about one-fifth the toxicity of mapharside.

3. Injected alone, Maph-BAL is more toxic than BAL.

4. Repeated doses of BAL (4 mg./kg.) are sufficient to save animals from the toxic effect of Maph-BAL.

5. Maph-BAL is more toxic to the ciliate *Colpidium colpoda* than mapharside.

6. The clinical implications of these facts are discussed.

We wish to express our thanks to Dr S. White of Parke Davis & Co. for a gift of pure mapharside, to Mr C. Dear for skilled assistance, and to Mr Wakelin for preservation of the strain of ciliate used.

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An Experimental Investigation of the 'Ring Hypothesis' of Arsenical Toxicity

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Earlier papers in this series (Stocken & Thompson, 1946*a*, *b*) described the development between 1940 and 1942 of British Anti-Lewisite (BAL) as an antidote for arsenical poisoning. It is now believed that the most important way in which the toxic arsenicals interfere with the normal life of the cell is by inhibiting an essential stage, that of pyruvate oxidation, in the series of chemical reactions by which it is supplied with energy (Peters, Sinclair & Thompson, 1946). The subsequent discovery (Stocken & Thompson, 1946*a*) that the arsenical protein formed by the interaction of lewisite and kerateine contains over 70% of its arsenic in combination with sulphur in the ratio of 1As:2S, suggested that the vesicant arsenicals act by reacting with two neighbouring essential thiol groups in enzyme proteins (e.g. those of the pyruvate system) with the formation of a relatively stable cyclic thioarsinite. The introduction into this system of a dithiol with which the arsenic can form a cyclic thioarsinite more stable than that which it forms with the protein, causes the arsenic to leave the protein and reverses its toxic effect on the cell. Monothiols, which, in the case of arsenical vesicants, are useless as antidotes, form open-chain thioarsinites which are less stable than the protein thioarsinites and are thus unable to remove the arsenic.

A confirmation of this hypothesis, which may be termed the 'ring hypothesis' of arsenical toxicity, has been sought in a study of the relative effectiveness as arsenical detoxicants of a series of $\alpha\omega$-dithiols and some monothiols with comparable physical properties. If the formation of a small stable ring is the underlying reason for the ability of dithiols to detoxicate arsenic *in vivo*, clearly the size of the ring is one of the most important factors in determining their therapeutic effectiveness. Attention was first directed to 1:2 and 1:3-dithiols because the corresponding cyclic thioarsinites are five- and six-membered rings, and by analogy with other types of ring compounds, rings of this size would be expected to be the easiest to form and among the most stable when formed; but it was thought desirable to undertake a study of the variation of antidotal efficiency attendant upon a successive increase in the spacing of thiol groups one from the other in the dithiol molecule in order to investigate the validity of the ring hypothesis still further. The relative effectiveness of the series of $\alpha\omega$-dithiols was assessed by measuring their ability to reactivate the pyruvate oxidase system after poisoning with lewisite. The results obtained are discussed later in the light of known facts about the stereochemistry of large rings and are considered to support the ring hypothesis.

EXPERIMENTAL

Synthesis of $\alpha\omega$-dithiols

The starting-point for the synthesis of most of the $\alpha\omega$-dithiols was the succinic acid series of dicarboxylic acids. These were esterified and reduced by the Bouveault-Blanc method (using Manske's procedure, 1934) to the corresponding diols, which were converted to the dithiols via the dibromides by heating the latter with 6 mol. ethanolic sodium or ammonium hydrosulphide (Stocken, 1947) at 80–90° in a sealed bottle for 24 hr. On cooling, the reaction mixture was acidified (<pH 4), the solvent removed *in vacuo* and the dithiol extracted from the solid residue with chloroform. After drying, the chloroform was removed

and the residual dithiol distilled *in vacuo*. Butanedithiol could not be prepared this way (cf. Schwarzenbach & Epprecht, 1936) and was obtained from the dibromide by a new method via the dithioacetate.

At the time this work was being carried out, only six members of the series of dithiols had been recorded. More recently, Hall & Reid (1943) have described the remaining members of the series up to dodecanedithiol. They, too, had to resort to an indirect method for butanedithiol. In the following account, experimental details are given only of the new compounds and method of preparation. These, like the other dithiols, are colourless oils with mercaptan-like odour, soluble in fat solvents, very sparingly soluble in buffer solutions at pH 7·3, and practically insoluble in water. When pure, they resist oxidation, and cannot be satisfactorily titrated with iodine, unless in very dilute (buffered) solution.

1:4-*Butanedithiol*. Dibromobutane (10 g.) was refluxed for 6 hr. with an excess of 10% (w/v) ethanolic sodium thioacetate (10 g.). The solution was filtered, evaporated, and 1:4-bis(*acetylthio*)*butane* was obtained by ether extraction of the residue (8·6 g.; yield 90%; b.p. 115°/0·8 mm. Found: S, 30·9. $C_8H_{14}O_2S_2$ requires 31·1%). The product (7·9 g.) was kept overnight with ethanolic NaSH (36 ml. of 12% (w/v)), and the reaction completed by warming. The thiol was obtained from the reaction mixture in the usual way (2·3 g.; yield 50%; b.p. 85–87°/15 mm. Found: S, 51·9. Calc. for $C_4H_{10}S_2$: S, 52·5%).

1:6-*Hexanedithiol*. Diethyl adipate (44 g.) was reduced by Manske's procedure to the glycol (18 g.; yield 80%; b.p. 142°/12 mm.), which was converted to the dibromide (31 g.; yield 81%; b.p. 102°/3 mm.). Treatment with ethanolic NaSH (12% (w/v)) gave the dithiol directly (yield 55%; b.p. 114°/13 mm. Found: S, 42·0. Calc. for $C_6H_{14}S_2$: S, 42·7%). The dibromide was also converted to the thiol indirectly (Found: S, 42·2%) via 1:6-bis(*acetylthio*)*hexane* (yield 81%; tablets, m.p. 29·5–30·5°; b.p. 137–138°/0·9 mm.; Found: S, 27·0; $C_{10}H_{18}O_2S_2$ requires S, 27·4%) using the method described above.

1:7-*Heptanedithiol*. Diethyl pimelate (22 g.) (Müller & Rölz, 1931) was reduced as above, and without isolating the glycol, fuming HBr (145 ml.) was added cautiously with strong cooling to the diluted (120 ml. water) and evaporated reduction mixture. The dibromide (9·8 g.; yield 37%; b.p. 130–132°/10 mm.) was isolated from the reaction mixture after refluxing about 15 hr. and was converted to the thiol (4·5 g.; yield 70%; b.p. 87–90°/1 mm.) with 15% (w/v) methanolic NH_4SH as already described. After extracting from chloroform by N-NaOH, and subsequently from the neutralized aqueous phase with fresh chloroform, and redistillation, the *dithiol* was pure. (Found: S, 39·2. $C_7H_{16}S_2$ requires S, 39·0%.)

1:8-*Octanedithiol*. Suberic and azelaic acids (Carmichael, 1922) were converted to dibromoctane (b.p. 137–140°/10 mm.) and dibromononane (b.p. 146–147°/13 mm.) as for the heptane homologue. The former yielded *octanedithiol* (0·9 g.; yield 58%, b.p. 101°/1 mm.) by the ammonium hydrosulphide method. (Found: S, 36·0. $C_8H_{18}S_2$ requires S, 36·0%.)

1:9-*Nonanedithiol*. This was prepared as for the previous homologue (4·8 g.; yield 74%; b.p. 103°/0·8 mm.). After two further distillations it was pure. (Found : S,33·1. $C_9H_{20}S_2$ requires S, 33·3%.)

1:10-*Decanedithiol*. M.p. 20°, b.p. 165–166°/15 mm. (Found: S, 31·1. Calc. for $C_{10}H_{22}S_2$: S, 31·1%.)

1:11-*Hendecanedithiol*. Ethyl 11-hydroxyhendecanoate (8·5 g.) (Walker & Lumsden, 1901) was reduced by Manske's procedure to the glycol (5·3 g.; yield 81%; prisms from benzene, m.p. 63°) which was converted via the dibromide (6·6 g.; yield 75%; b.p. 175–176°/10 mm.) to the *dithiol* (2·9 g.; yield 30%; b.p. 173°/13 mm. Found: S, 28·7; $C_{11}H_{24}S_2$ requires S, 29·1%) by heating with 20% methanolic NH_4SH for 3 days.

1:12-*Dodecanedithiol*. The glycol (3 g.; yield 61%; prisms, m.p. 66–67°, from benzene) from diethyl 1:10-decanedicarboxylic acid (7·3 g.) (Walker & Lumsden, 1901) was converted via the dibromide (3·8 g.; yield 76%; b.p. 186°/10 mm.) to the *dithiol* (1·5 g.; yield 50%; m.p. 22°, b.p. 155°/2 mm. Found: S, 28·1; $C_{12}H_{26}S_2$ requires S, 27·4%) by the same method.

Monothiols

For purposes of comparison some monothiols possessing similar properties to the dithiols were required.

n-Hexanethiol was prepared from the corresponding bromide using sodium hydrosulphide; b.p. 43·5–44°/14 mm. (Found: S, 26·6. Calc. for $C_6H_{14}S$: S, 27·1%.)

2-*Ethyl-n-hexanethiol* was prepared in a similar way; b.p. 66°/12 mm. (Found: S, 21·9. Calc. for $C_8H_{18}S$: S, 21·9%.)

Enzyme experiments

The pyruvate oxidase system has been used in this laboratory throughout the work on anti-arsenicals as a convenient test system for assessing antidotal efficiency. In the present work, the pigeon brain *brei* preparation was used (Passmore, Peters & Sinclair, 1933), the respiration of *brei* being measured manometrically at 38° and pH 7·3 by the Warburg technique in the presence of 0·02 M-sodium pyruvate and Ringer phosphate buffer.

Portions of *brei* (50–100 mg.) were transferred to the corked tared bottles containing pyruvate and buffer (final vol. 3 ml. after additions). After re-weighing and dividing the tissue with a blunt rod, lewisite (final conc. 16 μM) was added to all bottles (except a pair of controls); 10 min. later, each thiol to be tested (final conc. 24 μM) was added to a pair of lewisite bottles. Normally, one brain was divided between 6 pairs, enabling four thiols to be tested in each experiment. Preliminary experiments showed that the thiols alone, in this concentration and under these conditions, did not themselves inhibit the enzyme (Table 1). NaOH-papers were placed in position and duplicate readings taken at 30 and 60 min.

Solutions. Ringer phosphate buffer: NaCl, 7·2 g., KCl, 0·2 g.; $NaHCO_3$, 0·12 g.; KH_2PO_4, 13·8 g. in 1 l. glass distilled water with enough 20% NaOH to bring to pH 7·3. Sodium pyruvate (prepared by R. W. Wakelin by method of Peters, 1938): 0·2 ml. of 0·3 M in Ringer phosphate buffer per bottle. Lewisite: solutions of lewisite oxide were freshly prepared in glass distilled water, 0·1 ml. of 480 μM being used per bottle. This concentration gives an approximately 50% inhibition of total respiration very reproducibly (57%, σ 7·7 in 25 experiments).

Thiols: The $\alpha\omega$-dithiols are practically insoluble in water, and a number of preliminary experiments had to be carried out to devise a satisfactory method of introducing the desired concentrations into the bottles. Two final series of experiments were carried out. In the first, appropriate quantities of freshly prepared saturated solutions of the thiols in Ringer phosphate buffer, made by shaking a few mg. with 50 ml. buffer and filtering to remove oily droplets of undissolved thiol, were added to the bottles after standardization with N/500 iodine. This was unsatisfactory, as thiols above nonanedithiol were too insoluble and there was the possibility of oxidation. A second series was accordingly performed by adding the thiols in 0·025 ml. *iso*propanol (usually 0·05 ml. of a solution of twice the desired strength diluted with an equal volume of buffer just before addition). *Iso*propanol has practically the same vapour pressure as water (Dixon & Needham, 1941) and in this concentration inhibits the enzyme to the extent of about 17 % (Table 2). This was allowed for by adding equal amounts of the alcohol to the control and lewisite bottles. Solutions of monothiols were made up similarly.

The final concentration of the dithiols was 24 μM (monothiols, 48 μM) giving a thiol/lewisite ratio of 1·5 (monothiols, 3).

Expression of results. If x, y, z are the average oxygen uptakes in μl./g. wet tissue/hr. of the bottles without additions, with lewisite, and with lewisite and thiol respectively, the inhibitions I_0 and I, due to lewisite, alone, and in the presence of thiol, are $I_0 = 100\frac{x-y}{x}$ and $I = 100\frac{x-z}{x}$. The percentage reactivation is then defined as $100\frac{I_0-I}{I_0} = 100\frac{z-y}{x-y}$.

RESULTS

Effect of dithiols and iso*propanol on enzyme.* Table 1 shows that the dithiols themselves, in the very low concentrations used in these experiments are without appreciable toxic effect. Table 2 gives the results of two control experiments with *iso*propanol.

Relative efficiency of thiols. Table 3 summarizes the results of the two series of experiments. It will be seen, first, that the monothiols are incapable of reversing, to any significant extent, the toxic action of lewisite (confirming earlier unpublished protection experiments by Sinclair with other monothiols) and second, that there is a definite variation in the antidotal efficiency of the dithiols with the distance apart of the thiol groups in the aliphatic chain. The implications of this result for the ring hypothesis are discussed later.

Table 1. *Effect of small concentrations of dithiols on pigeon brain pyruvate oxidase*

(Numbers of experiments given in brackets in col. 3.)

Thiol	Concentration (μM)	Inhibition over 60 min. (%)
Ethanedithiol	25	0 (2)
Pentanedithiol	24·5	0 (1)
Heptanedithiol	24	9 (1)
Octanedithiol	30	0 (2)
Nonanedithiol	24	0 (1)

Table 2. *Effect of* iso*propanol on pigeon brain pyruvate oxidase*

*Iso*propanol (ml.)	O_2 uptake in 60 min. (μl./g. tissue) Control	Alcohol	Inhibition (%)
0·025	1563	1295	17
0·05	1504	1225	19

Table 3. *Reactivation of pigeon brain pyruvate oxidase by thiols* 10 *min. after poisoning with lewisite*

(Lewisite, 16 μM; dithiols, 24 μM; monothiols, 48 μM.)

Thiol	Reactivation over 60 min. (%) Thiols added in buffer	Thiols added in 0·025 ml. *iso*propanol	Mean of all exp.
Ethanedithiol	100, 93	100, 97	98
Propanedithiol	73, 75	81*, 79	77
Butanedithiol	18	51, 9, 18, 33	26
Pentanedithiol	51, 66, 36	68*, 85, 43, 49	57
Hexanedithiol	84, 98	94, 88	91
Heptanedithiol	83, 76, 95	98,93	89
Octanedithiol	70, 86, 86	100, 87, 91, 94	88
Nonanedithiol	83, 87, 79	93*, 98	88
Decanedithiol	—	100, 100	100
Hendecanedithiol	—	85, 69	72
Dodecanedithiol	—	80, 75	78
Butanethiol	3	7	5
Hexanethiol	4	20	12
Heptanethiol	7	0	4
2-Ethylhexanethiol	—	20	20

* 0·05 ml. *iso*propanol.

Table 4. *Detoxication of lewisite by thiols in the presence of pigeon brain pyruvate oxidase*

(Lewisite, 16 μM; dithiols, 24 μM; monothiols, 48 μM.)

Thiol	Detoxication over 60 min. (%)
Ethanedithiol	99
Propanedithiol	95
Butanedithiol	74
Pentanedithiol	95, 95
Hexanedithiol	91, 100
Heptanedithiol	100, 100
Octanedithiol	100, 100
Nonanedithiol	100
Butanethiol	6
Hexanethiol	4
Heptanethiol	10

Detoxication experiments. In another series of experiments, the thiol and lewisite (added in buffer)

were allowed to react for 30 min. before addition of the brain preparation, and the percentage detoxication (defined by the same equations as the reactivation) measured. Table 4 shows that the difference between the monothiols and dithiols is as marked as before, but that under these much less critical conditions, there is a less marked variation among the various dithiols.

Rate of oxidation of dithiols

A possible objection to any arguments based on the variations in antidotal efficiency of the $\alpha\omega$-dithiols revealed in Table 3 is that the observed differences are merely due to variations in the ease of oxidation of the dithiols. This is unlikely on theoretical grounds as there would be little tendency for oxidation to occur either (*a*) in *iso*propanol, or (*b*) after combination with arsenic. Thus, although oxidation might have occurred in the buffer solution before the experiments in the first series, thereby reducing the effective concentration of the more easily oxidized dithiols, this would be unlikely to occur in the *iso*propanol solutions of the second series; and the good agreement in general tendency between the two series makes it also unlikely that it has in fact occurred in the first. Moreover, if during the experiments the varying efficiency of the different thiols is not due to a genuine variation in ability to remove the arsenic from the protein, but due to a variation in the rate of oxidation after the arsenic has been removed, it would be necessary to assume that thiols are readily oxidized while in combination with arsenic, which is not supported by the properties of such cyclic thioarsenites as have been obtained pure. In order to put the matter beyond doubt, some comparisons were made between the rate of oxidation of butane and pentane dithiol, which have a low antidotal effect, and ethane and hexane dithiol, which are fully effective in the concentrations used.

Appropriate quantities of the dithiols in 0·1 ml. *iso*propanol were added to 200 ml. M/15 phosphate buffer (pH 7·3) to give an initial concentration of 0·5 mM. Of this 25 ml. samples were withdrawn at intervals, acidified and titrated with N/200 iodine. Curve *A* (Fig. 1) shows the disappearance of ethanedithiol in the absence of aeration; the solution rapidly falls to three-fifths of its original value, and thereafter oxidizes only very slowly, the rate of diffusion of oxygen into the solution probably being the limiting factor. In curve *B* the results of several experiments with ethane, butane, pentane and hexane dithiols are plotted. Air was drawn through the solutions during the course of the reaction and the curve is now of the ordinary unimolecular form. It will be seen that all the thiols follow essentially the same curve (K approx. 0·02 min.$^{-1}$) and the variations in the antidotal efficiency of the dithiols cannot simply be attributed to varying ease of oxidation.

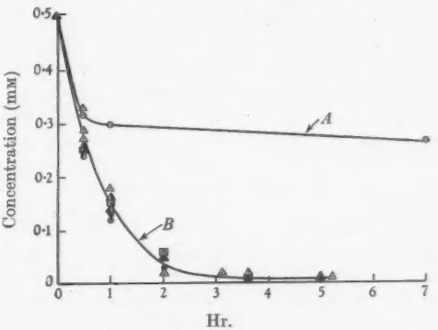


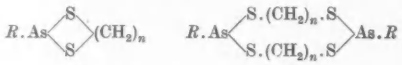
Fig. 1. Oxidation of dithiols. *A*, ⊙ ethanedithiol (without aeration); *B*, ⊡ ethanedithiol, ◐ butanedithiol, ◑ pentanedithiol, △ hexanedithiol (with aeration).

DISCUSSION

The most striking of the results recorded above is the superiority of the dithiols as a class over the monothiols, both in the reactivation and in the protection experiments. The most natural explanation of this is that given by the ring hypothesis. On this view, all the $\alpha\omega$-dithiols form cyclic thioarsinites of a stability greater than, or comparable to, that of the lewisite-enzyme compound, the individual variations in effectiveness being due to the varying stabilities of the thioarsinite rings. The monothiols, on the other hand, form open-chain thioarsinites less stable than the protein thioarsinite, and are thus without effect.

Nevertheless, it is evident that beyond a certain point, the thiol groups will be spaced so far apart in the dithiol molecule that even the cyclic thioarsinite formed by the arsenic with the antidote will be less stable than that formed with the protein. In spite of the unfavourable properties of the dithiols above decanedithiol, which may preclude effective penetration to the active centres of the brain enzymes, it is suggested that such a point may have been reached with hendecane and dodecanedithiol, which are less effective in reactivating the enzyme after poisoning (Table 3). One previous indication has indeed been obtained that stereochemical factors may limit the effectiveness of dithiols as antidotes; thus Thompson found that 2:6-dimercaptopyridine-4-carboxylic acid was unable to protect pyruvate oxidase against lewisite (Stocken & Thompson, 1943). In the writer's opinion, a crucial test would be provided by a comparison of the antidotal effectiveness of *cis-* and *trans*-1:2-*cyclo*pentanedithiol, but so far this has not been attempted.

Table 3 shows that in addition to the falling off in effectiveness at the end of the series, there is a pronounced minimum in the middle of the range, butane and pentane dithiols being the least effective of any. The high dilutions used in these experiments (about 20 μM) make it very likely that if rings are formed, they will be rings of type 1 and not dimeric or more complex rings (cf. 2):



(1) (2)

It is, then, on the stability of these rings in aqueous solution that the effectiveness of the dithiol as an antidote depends. The results show that the least stable ring is that corresponding to butane-dithiol, i.e. the seven-membered ring; the eight-membered ring is intermediate in stability between this and the rings of smaller and greater size, containing 5, 6, 9, 10, 11, 12 and 13 atoms, which are all sufficiently stable to prevent the arsenic acting on the enzyme protein. The rings greater than this again show a decline in their stability. In the absence of any detailed information as to the steric factors involved in the combination of arsenic with the enzyme protein, it is not possible to deduce directly the probable stability of the protein-arsenic compound, or the size of the ring formed, but the reactivation experiments suggest that its stability lies between those of eight- and fourteen-membered thioarsinite rings.

It should perhaps be emphasized that the 80–100 % reactivation obtained with all the dithiols from hexane to decane dithiol does not make it necessary to assume that their cyclic thioarsinites are as stable as those derived from ethane and propane dithiol and containing only five- and six-membered rings. This is illustrated diagrammatically in Fig. 2. Curve *A* shows the expected variation of stability with ring size; the horizontal line *B* represents the stability of the protein thioarsinite. Any dithiol whose thioarsinite has a stability corresponding to a point above *B* will be able to effect maximum, or nearly maximum, reactivation, though its stability may fall very much short of the maximum; conversely, a dithiol corresponding to a point below *B* will give a much lower reactivation, though, from the protection experiments, the curve *A* will at no point lie far below *B*.

Our knowledge of large rings is chiefly due to Ruzicka and his school (Ruzicka, 1926). It is well known that the yields of cyclic ketones and lactones fall off rapidly as the ring size increases beyond six, and then rise again with increasing size. In Ruzicka's work, the improvements in yield are small; but in Zeigler's experiments, in which he used his high dilution technique, the minimum in the curve relating yield and ring size is much more pronounced (cf. Zeigler, 1934).

Stoll & Stoll-Compte (1930) have investigated the stereochemistry of large ring formation and have explained this drop with 7–9-membered rings in terms of a change in the packing of the atoms of the

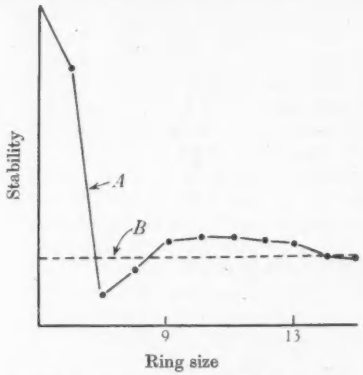


Fig. 2. Expected relative values for the stability of thioarsinites derived from dithiols and enzyme protein. *A*, cyclic thioarsinites; *B*, protein thioarsinite.

ring as the size increases. This region of minimum yield corresponds to a change from an open ring to a ring large enough to consist essentially of two parallel aliphatic chains lying side by side, joined at the ends, in which the hydrogen atoms are no longer forced to the outside of the ring but can be accommodated in the same positions as they would occupy in an aliphatic chain. This change in the spatial arrangement of the atoms is reflected in a change in molecular density and it is therefore significant that the Stolls were able to correlate changes in density of the ring ketones with changes in yield.

This work offers an explanation of why the ease of formation of rings changes with ring size even in a region where multiplanar strainless rings can exist. The Stolls's arguments are supported by the work of Katz & Selman (1928) who showed by X-ray examination that the larger rings do in fact exist in a collapsed condition.

So far, large rings containing metal or metalloid atoms have not been prepared in a pure state, and until this is done, and a systematic study made of their physical properties, an application of the Stoll-Ruzicka theory of large rings to the present problem can only be tentative. Nevertheless, the same general considerations very probably apply to the cyclic thioarsinites, if account be taken of the fact that S and As atoms are not quite equivalent stereochemically to methylene groups. The difference lies less in their different valency angles

than in the absence of the two hydrogen atoms, which will significantly increase the amount of space available for the packing of the other hydrogen atoms. It is to this that the difference between the extent of the minimum in the antidotal efficiency of the dithiols and the minimum of the carbocyclic rings must be ascribed. Furthermore, in the case of rings like the thioarsinites, which can be opened by hydrolysis and whose stability in aqueous solutions is therefore bound up with their ease of formation, it is evident that the Stoll-Ruzicka theory, though originally applying to ring formation, can here be equally applied to ring stability.

It remains to dispose of a possible alternative explanation of the characteristic form of the results of Table 3. It might be maintained that the antidotal efficiency of the higher dithiols was not due to ring formation at all, but merely to the excessively small solubility which is in all probability a feature

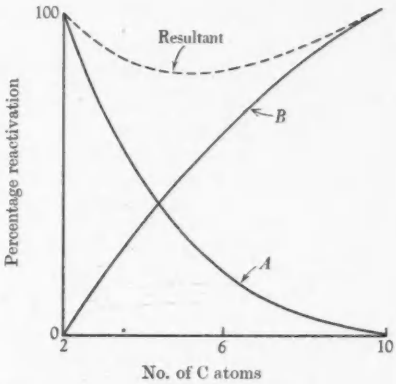


Fig. 3. Possible factors in reactivation by $\alpha\omega$-dithiols. *A*, steric factor; *B*, solubility factor.

of their thioarsinites. On this view, the minimum is to be explained as a result of two contrary factors, as illustrated in Fig. 3, the first (curve *A*), the decreasing efficiency of the dithiols due to the successive increase in the distance separating the two thiol groups; the second (curve *B*), the increasing efficiency due to the increasing insolubility of the thioarsinites.

It would seem that this alternative explanation is excluded by the very poor degree of reactivation (and protection) obtained from the monothiols. It is difficult to see why a dithiol like octane-dithiol should be so efficient and a monothiol like 2-ethyl-*n*-hexanethiol, possessing very similar physical properties, should be so inefficient, unless ring formation is the mechanism involved; and if it is, the rise in antidotal efficiency after butanedithiol must be due to the stereochemical effects outlined above, because physical properties show a steady gradation throughout the series.

It may, therefore, be concluded that the results of Table 3 and in particular the falling off in efficiency with which butane and pentane dithiol reverse the effect of lewisite, is not inconsistent with the ring hypothesis, but is what might be expected from it after a careful consideration of the finer points in the stereochemistry of the larger rings.

SUMMARY

1. A series of $\alpha\omega$-dithiols from ethane to dodecane dithiol has been synthesized and their relative antidotal efficiency has been measured and compared with that of some monothiols with similar physical properties, using pigeon brain pyruvate oxidase as an *in vitro* test system.

2. The results, when discussed in the light of known facts relating to the stereochemistry of large rings, are found to support the 'ring hypothesis' of arsenical toxicity.

The work described in this paper was undertaken as part of a research programme for the Chemical Defence Research Department of the Ministry of Supply under the direction of Prof. Peters.

I should like to express my gratitude to the Chief Scientific Officer, Ministry of Supply, for permission to publish, and to Dr R. H. S. Thompson, and Dr L. A. Stocken for much help and encouragement. In particular my thanks are due to Dr Stocken for specimens of ethane, propane and pentane dithiols and two monothiols, to Dr Thompson for help in some of the enzyme experiments, and to the N.R.D.C. of the U.S.A. for other thiol samples. I am also grateful to Miss Kempson for technical assistance and to Mr Vincent for various pieces of glass apparatus.

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The Oxidation of Dithiols

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Warburg & Sakuma (1923) demonstrated that cysteine in aqueous solutions was not oxidized by atmospheric oxygen in the absence of heavy metals, and Harrison (1924) showed that this was true also for glutathione. Both SH-containing substances are sluggish oxidation-reduction systems, non-autoxidizable, and oxidized only in the presence of heavy metals or of iron porphyrins. The synthesis of 2:3-dimercaptopropanol (BAL) (see Peters, Stocken & Thompson, 1945) and the preparation of a large number of dithiols by Salzberg, Lazier, Signaigo & Pavlic (personal communication) made it necessary to study the conditions for the oxidation of the SH groups contained in these compounds as an essential step in effecting their stabilization. It will be seen from the experiments reported in this paper that these dithiols behave like the monothiols cysteine and glutathione as regards oxidation, i.e. they are sluggish non-autoxidizable oxidation-reduction systems, easily oxidized by catalytic amounts of copper and iron porphyrins. The spatial arrangement of SH and the addition of different groups to the molecule have made possible a study of the influence of these factors on the rate of oxidation.

EXPERIMENTAL AND RESULTS

Oxidation of 2:3-*dimercaptopropanol by copper.* The lack of oxidation of 2:3-dimercaptopropanol (dithiol 1 or BAL) in the absence of heavy metals could not be demonstrated conclusively because neither the compound itself nor the salts used in the preparation of the buffers were freed of traces of heavy metals present as impurities. However, the facts that the rate of oxidation was considerably diminished when buffer solutions were prepared with quartz-distilled water and kept in paraffin covered containers and that the rate of this oxidation was still further diminished on addition of cyanide are indications that the dithiol is oxidized only by the catalytic action of heavy metals. In 0·05 M-phosphate buffer pH 7·0 and 7·1 copper was the most powerful oxidizing agent among the heavy metals which act as catalysts for the oxidation of thiol compounds (Table 1). In these experiments,

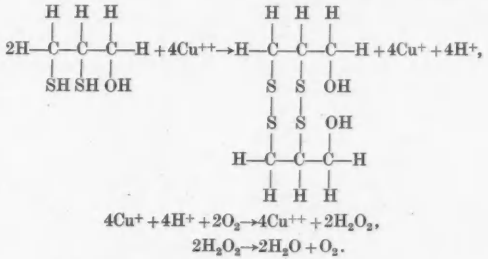
Table 1. *The oxidation of* 2:3-*dimercaptopropanol by atmospheric oxygen*

(Metal catalyst, 2×10^{-4} mmol.; dimercaptopropanol 1×10^{-2} mmol.; buffer, 0·05 M-phosphate; pH 7·1; temp. 38°.)

Added substance	Half-oxidation time (min.)
None	70·4
HCN (5×10^{-4} M)	123·0
$CuCl_2$	8·8
Cu-glycine (glycine, 0·1 M)	8·0
Cu-albumin (6% (w/v) albumin)	7·1
$FeCl_3$	42·5
$MnSO_4$	70·4
$CoSO_4$	70·4
$NiSO_4$	70·4

the amount of oxidizing catalyst was 2×10^{-4} mmol. while that of dithiol was 1×10^{-2} mmol. In the absence of added heavy metal, half-oxidation occurred in 70·4 min., being lengthened to 123 min. on addition of 5×10^{-4} M-HCN. Addition of $CuCl_2$ increased the rate more than seven-fold so that half-oxidation took place in 8·8 min. and with $FeCl_3$ in 42·5 min. $MnSO_4$, $CoSO_4$ and $NiSO_4$ had no effect. In the oxidation of ascorbic acid by Cu^{++} as catalyst, non-ionized copper had little catalytic action (Barron, DeMeio & Klemperer, 1936); the state of the copper had no influence on the rate of oxidation of BAL, for the rate remained unchanged on formation of copper complex compounds with alanine or egg albumin. Dimercaptopropanol might, therefore, be oxidized by the copper contained in biological fluids. In all these experiments the rate of oxidation was determined by measuring the O_2 uptake manometrically. The oxidation was complete with an uptake of one oxygen atom/mol. of dithiol, the insoluble disulphide complex being formed. Copper

acts as a catalyst by its reduction to Cu^{+} by the dithiol and reoxidation to Cu^{++} by oxygen. Whether the oxidation of the dithiol is a direct oxidation or whether there is previous formation of a metal complex, as with cysteine and iron, is not known. However, the lack of oxidation of hexane-1:6-dithiol, where the SH groups are separated by four carbon atoms, speaks in favour of the transitory formation of a cyclic Cu complex. The oxidation by copper may be represented as follows:



$$4Cu^{+} + 4H^{+} + 2O_2 \rightarrow 4Cu^{++} + 2H_2O_2,$$

$$2H_2O_2 \rightarrow 2H_2O + O_2.$$

Dimercaptopropanol is also oxidized by ferricyanide requiring 2 mol. of ferricyanide/mol. of dithiol. This oxidation was performed manometrically in a solution of 0·1 M-$NaHCO_3$ with N_2 and CO_2 as the gas phase. Under such conditions 2 mol. CO_2/mol. dithiol oxidized were formed. The manometric method can, in fact, be used for the determination of dimercaptopropanol, with the dithiol solution in the main vessel and 0·5 ml. of 10 % (w/v) $K_3Fe(CN)_6$ in the side arm. At 38° the reaction is complete in 3 min. Ferricyanide oxidation can also be used for the colorimetric estimation of dithiols by spectrophotometric measurement at 6900 A., of the ferrocyanide formed as prussian blue. The colorimetric method will measure from 3 to 20 μg. of dithiol. The temperature coefficient for the oxidation of dimercaptopropanol with Cu^{++} as catalyst was $Q_{10} = 1{\cdot}38$. From these data the value of the energy of activation, E, was calculated to be 9000 cal./mol. This low value indicates that the reoxidation of Cu^{+} is the reaction governing the rate of oxidation of dimercaptopropanol. That the temperature coefficient was about the same for the 'autoxidation' of dimercaptopropanol further indicates that this oxidation is due to heavy metal catalysis.

Oxidation of dithiols with iron porphyrins. Iron protoporphyrin (blood haemin) is a powerful catalyst for the oxidation of cysteine (Krebs, 1929) and of glutathione (Lyman & Barron, 1937). It has been found to be a more powerful catalyst for the oxidation of the dithiols 1, 2, 3 and 4 (Table 2). (The structural formulae of these dithiols are shown in the Glossary, p. 940.) The first three dithiols were oxidized with great speed. The oxidation of dithiol 4, which was extremely slow with $CuCl_2$ as catalyst, was 30 times as fast with haemin. When dithiols were added to a solution of haemin in phosphate buffer, there was immediate reduction of haemin, which was reoxidized by atmospheric oxygen. At the end of 3 hr., as a result either of the action of H_2O_2 produced during the oxidation of dithiol or of the action of the oxidized dithiol, there was destruction of the haemin molecule with opening of the porphyrin ring and disappearance of the typical Soret band at 3850 A. (Fig. 1). When dithiol was added to a solution of oxyhaemoglobin (dithiol 1, 1×10^{-3} M; oxyhaemoglobin, 2×10^{-5} M), there was also complete destruction of oxyhaemoglobin at the end of 2 hr. with disappearance of the absorption bands at 5415 and 5760 A. and marked decrease of the Soret band at 4150 A. (Fig. 2). The end-product is the so-called pseudohaemoglobin, a precursor of bilirubin. That this destruction of the iron-porphyrin molecule occurs only *during the continuous oxidation of dithiol* was demonstrated by the following two experiments: (1) 1×10^{-3} M-dithiol was added to 5×10^{-5} M-cytochrome *c*. There was immediate reduction of cytochrome, which remained reduced and without further alteration for 5 hr., the duration of the experiment; (2) 1×10^{-3} M-dithiol (saturated with H_2) was added to 2×10^{-5} M-haemoglobin (saturated with H_2). The extinction coefficients measured at 5525, 7550 and 7575 A., remained practically unchanged at the end of 2 hr.

When dithiol 1 (1×10^{-3} M) was added to methaemoglobin (2×10^{-5} M) there was immediate reduction to haemoglobin, which in the presence of

Table 2. *Oxidation of dithiols by atmospheric oxygen with iron protoporphyrin*

(Catalyst, blood haemin (0·0002 mmol.); dithiol (0·01 mmol.); pH 7·1; temp. 38°; vol. 3 ml.)

Dithiol*	Half-oxidation time (min.)
1 (BAL)	5·0
2	6·0
3	3·0
4	13·0

* See Glossary for nomenclature of dithiols.

dissolved oxygen was oxygenated to oxyhaemoglobin. This immediate reduction of methaemoglobin by dimercaptopropanol can be utilized for the treatment of methaemoglobinaemia.

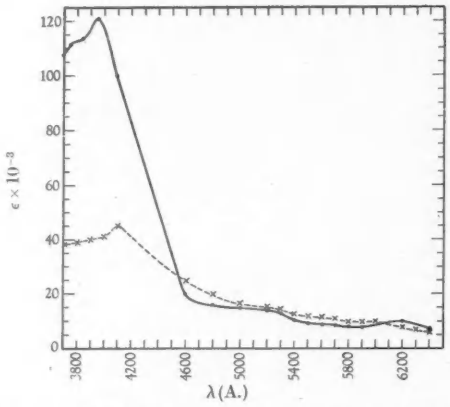


Fig. 1. Effect of BAL (1×10^{-3} M) on haemin ($4{\cdot}61 \times 10^{-6}$ M). Solid line, haemin; broken line, haemin + BAL.

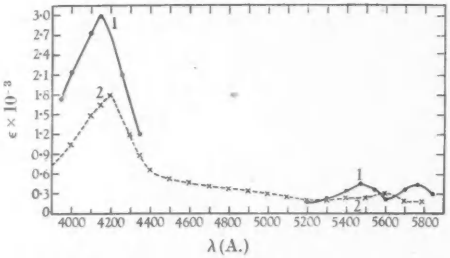


Fig. 2. Effect of BAL on oxyhaemoglobin. Spectrophotometric measurements 2 hr. after addition of BAL. 1, oxyhaemoglobin, 5×10^{-5} M; 2, oxyhaemoglobin + 10^{-3} M-BAL after 2 hr.

Webb & van Heyningen (1943) found that dithiol 1 reduces cytochrome *c* and is oxidized by the system cytochrome *c*-cytochrome oxidase. These findings have been confirmed. The initial rate of oxidation of dithiol did not increase on addition of cytochrome oxidase (prepared according to Haas, 1943); on addition of cytochrome *c* (2×10^{-5} M) and cytochrome oxidase, dithiol was rapidly oxidized (Fig. 3). All these experiments were performed in aqueous solutions with 0·02 M-phosphate buffer pH 7·4.

Dithiol 1 *and ascorbic acid.* It is known that addition of a sluggish oxidation-reduction system of low potential to a system of higher potential protects the latter from oxidation (protection of adrenaline from air oxidation by ascorbic acid, of ascorbic

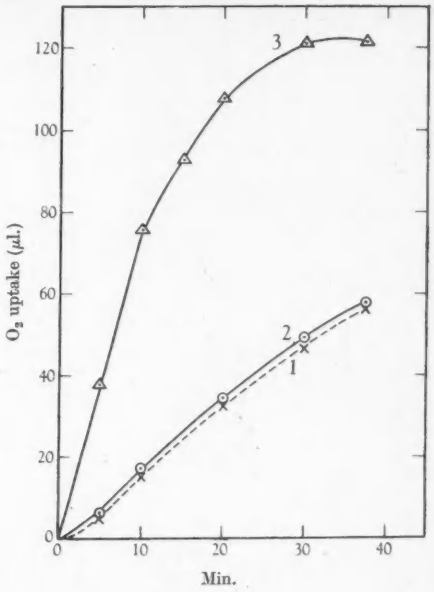


Fig. 3. Oxidation of BAL with cytochrome *c* and cytochrome oxidase. 1, BAL; 2, BAL + enzyme; 3, BAL + enzyme + cytochrome *c*.

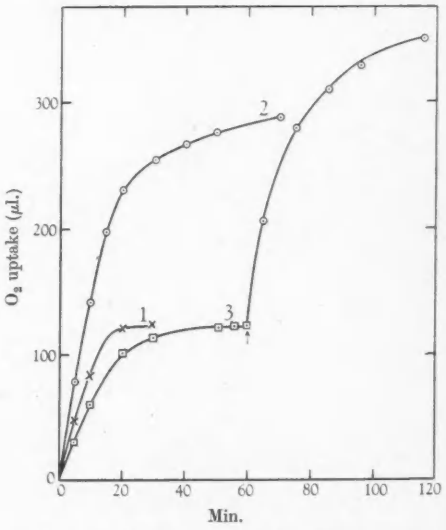


Fig. 4. Protection of ascorbic acid oxidation by BAL. $CuCl_2$, 2×10^{-4} mmol. 1, BAL; 2, ascorbic acid; 3, BAL + ascorbic acid (ascorbic acid oxidase added at arrow).

acid by glutathione). In the same manner dithiol 1 protects ascorbic acid from oxidation. The experiments were performed in 0·02 M-phosphate, pH 7·1 and 38°. The amount of dithiol was 1×10^{-2} mmol.; of ascorbic acid, 2×10^{-2} mmol.; of $CuCl_2$, 2×10^{-4} mmol. (at this concentration of copper and pH value, half-oxidation of ascorbic acid occurs in 10 min. (Barron *et al.* 1936). The oxygen uptake in the vessels containing dithiol alone, and dithiol plus ascorbic acid was the same, showing that ascorbic acid was not oxidized. At the end of 60 min., ascorbic acid oxidase prepared from squash was added to the vessel from the side arm and there was immediate oxidation of ascorbic acid (Fig. 4).

Reaction of 2:3-dimercaptopropanol in aqueous solutions with heavy metals. Dimercaptopropanol, like many other thiol compounds, reacts with heavy metals and gives in many instances almost insoluble coloured compounds. Coloured compounds are formed with Fe^{+++} (blue-green), Fe^{++} (light pink), Pb (green-yellow), Sn (yellow), Bi (green-yellow), Cu^{++} (dark blue), Co (brown), Ni (brown turning to green in NH_4OH), Sb (brown to white precipitate), Se (yellowish white precipitate). Zn, Cd and Hg^{++} give white precipitates. Mg and Mn give neither coloured reaction nor precipitate. The combination of Mg with dimercaptopropanol with formation of a soluble complex seems to be the reason for the inhibition produced by $MgCl_2$ on the rate of oxidation of dithiol. When the rate of oxidation of dithiol with $CuCl_2$ as catalyst was measured in the presence and in the absence of 0·01 M-$HgCl_2$, it was found that Mg inhibited the rate of oxidation by 44 %.

Oxidation of other dithiols with copper as catalyst. The position of the SH groups and the nature of the residues attached to the dithiols seem to be an important factor in determining the efficiency of oxidation catalysts like Cu^{++}. To study the relative importance of these factors the rates of oxidation of 20 different dithiols and one trithiol with and without added copper as catalyst were determined. Copper failed to catalyze appreciably the rate of oxidation in four of the compounds tested (dithiols 3, 4, 6 and 7) and had very little effect on the oxidation of 1:2:3-propanetrithiol.

The rate of oxidation of dithiols, having the SH groups contiguous to each other, varied as would be expected according to the nature of the attached residue. Thus while dithiol 1 was half-oxidized in 10·5 min. at 22·4° and pH 7·0, a closely related compound, dithiol 21 with one C atom more in the residue was half-oxidized in 22 min. By replacing the last C groups with an acid group, as in dithiol 10, the rate of oxidation was diminished still further so that half-oxidation occurred in 55 min. Attachment of a methyl ester group to this acid, as in dithiol 11, increased the rate of oxidation sufficiently to eliminate the inhibitory effect produced by the carboxyl group; in fact, half-oxidation occurred in 10·4 min., the same as that of dithiol 1. A progressive increase in the number of C atoms in the molecule, in the form of the ethyl, *n*-propyl and *n*-butyl esters of dithiol 10 (dithiols 8, 18, 19) resulted in an increase in the time necessary for the half-oxidation of each compound. Thus the ethyl ester (dithiol 8) was half-oxidized in 13·5 min.; the propyl ester (dithiol 18), in 23·6 min. and the butyl ester (dithiol 19) in 123 min. The ether derivatives of dithiol 1 behaved like the esters. The methyl ether (dithiol 13) was half-oxidized in 15 min. (much faster than the free acid dithiol 10). Increasing the length of the chain resulted in an increase in the half-oxidation time of each compound: the ethyl ether (dithiol 9) was half-oxidized in 17 min.; and the *iso*propyl ether (dithiol 20) in 25·6 min. The urea and carbamate derivatives of dithiol 1, had a rate of oxidation only slightly slower than that of dithiol 1. Thus dithiol 14 was half-oxidized in 15·5 min. and dithiol 15 in 21 min. The rate of oxidation of phenylethane dithiol (dithiol 17) was six times slower than that of dithiol 1, while the *N*-phenylamine derivative (dithiol 7) was hardly oxidized at all. The only compound which had a rate of oxidation slightly faster than that of dithiol 1 was dithiol 12, which was half-oxidized in 8 min. The addition of another SH by substitution of the OH group of dithiol 1, thus making propanetrithiol (dithiol 5), had the effect of slowing down the rate of oxidation. Propanetrithiol was half-oxidized in 127 min., i.e. 12 times

Table 3. *Oxidation of dithiols by atmospheric oxygen with* $CuCl_2$ *as catalyst*

(Each vessel contained 0·03 M-phosphate buffer pH 7·0; $CuCl_2.2H_2O$ (2×10^{-4} mmol.); dithiol (0·01 mmol.); vol. 3·0 ml.; temp. 22·4°.)

Dithiols*	Half-oxidation time (min.)	
	Copper added	No copper
1	10·5	196
2	25·5	500
3	350	500
4	500	500
5	127	224
6	500	500
7	500	500
8	13·5	500
9	17·0	106
10	55·0	500
11	10·4	80
12	8·0	176
13	15·0	101
14	15·5	176
15	21·0	177
16	27·0	500
17	65·0	312
18	23·6	500
19	123·0	500
20	25·6	116
21	22·0	240

* See Glossary for nomenclature of dithiols.

as slowly as dithiol 1. Dithiol 16, the *bis*-S (*N*-ethylacetamidomethyl) ether of dithiol 1 has been reported to split in solution, and yield dithiol 1 quantitatively (Dr Lazier, personal communication). The resulting dithiol should then be oxidized at a rate similar to that of dithiol 1. This was the case, for dithiol 16 did actually undergo a fairly rapid rate of oxidation in the presence of copper (Table 3).

Separation of SH groups by one C atom and an OH residue between the SH groups (dithiol 2) increased the half-oxidation time to 25·5 min. Substitution of H for OH on the middle carbon atom (dithiol 3) inhibited almost completely the oxidation of the dithiol. Further separation of the SH groups, as in dithiol 4 where the SH groups are separated by 4 carbon atoms, retarded oxidation almost completely.

Effect of pH *on the rate of oxidation.* The pK value of the SH group of glutathione is 9·62 (Pirie & Pinhey, 1929). The rate of oxidation of glutathione would therefore increase as the SH group dissociates (more alkaline pH), and decrease when undissociated (more acid pH). Lyman & Barron (1937) showed, in fact, that the rate of oxidation of glutathione varied markedly with the pH of the medium. There is the same influence of the hydrogen-ion concentration on the rate of oxidation of dithiols. Table 4 shows as an example the effect of pH on the oxidation of dithiol 15. At pH 4·0 there was no oxidation; at pH 6·0 the half-oxidation time was 109 min.; at pH 9·4 it was only 6·5 min.

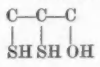
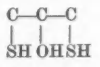
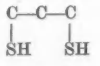
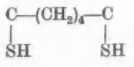
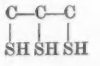
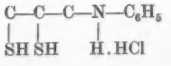
Table 4. *Effect of* pH *on the oxidation of dithiol* 15 *by atmospheric oxygen and* Cu^{++} *as catalyst*

(Each vessel contained 0·03 M-buffer (pH 9·4, borate buffer; pH 6·0, 7·0, 8·0, phosphate buffer; pH 5·4, 4·0, acetate buffer); $CuCl_2.2H_2O$ (2×10^{-4} mmol.); dithiol 15 (0·01 mmol.); total vol. 3·0 ml.; temp., 20°.)

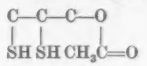
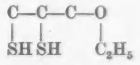
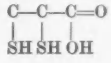
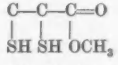
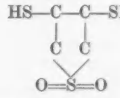
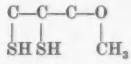
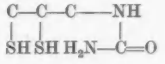
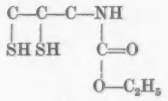
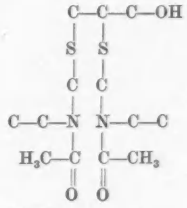
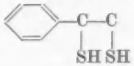
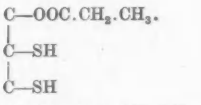
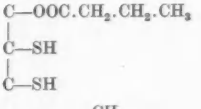
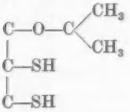
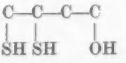
pH	Half-oxidation time (min.)
9·4	6·5
8·0	15·5
7·0	20·5
6·0	109·0
5·4	300·0
4·0	No oxidation

The oxidation-reduction potential of dithiol 1. In electro-active systems there is, under certain conditions, a relation between the oxidation-reduction potential and the rate of oxidation. Whether such a relation exists also in sluggish systems seems doubtful because there must be in these systems a greater difference between the ΔF and the ΔF^* (free energy of formation of the activated state) values of the system. The E'_0 of dithiol 1 was determined at pH 7·0 in equimolecular mixtures of the oxidized and reduced component with cresyl violet as the electro-active mediator. It was found to be $-0{\cdot}150$ V. It was not possible to determine the E'_0 values of the other dithiols.

GLOSSARY

Compound	Name	Skeleton formula
1	2:3-Dimercaptopropanol (BAL, DTH)	
2	1:3-Dimercaptopropanol	
3	Propane-1:3-dithiol	
4	Hexane-1:6-dithiol	
5	Propane-1:2:3-trithiol	
6	Naphthalene-1:5-dithiol	
7	*N*-Phenyl-aminopropanedithiol-HCl	

GLOSSARY (*cont.*)

Compound	Name	Skeleton formula
8	2:3-Dimercaptopropyl acetate	
9	2:3-Dimercaptopropyl ethyl ether	
10	2:3-Dimercaptopropionic acid	
11	Methyl 2:3-dimercaptopropionate	
12	3:4-Dimercaptotetrahydrothiophene-1-dioxide	
13	2:3-Dimercaptopropyl methyl ether	
14	2:3-Dimercaptopropyl urea	
15	*N*-(2:3-Dimercaptopropyl) carbamate	
16	bis-*S*(*N*-Ethylacetamidomethyl) ether of BAL	
17	Phenylethanedithiol	
18	2:3-Dimercaptopropyl propionate	
19	2:3-Dimercaptopropyl butyrate	
20	2:3-Dimercaptopropyl *iso*propyl ether	
21	3:4-Dimercaptobutanol-1	

DISCUSSION

Of the 20 dithiol compounds only one, dithiol 12, was oxidized with copper as a catalyst at a rate slightly faster than that of dithiol 1. Dithiol 11 was oxidized at the same rate. The rate of oxidation of all the others was slower. The effect of various groupings on the rate of oxidation of the different dithiols is mainly determined by the electron attracting powers (electronegativity) of the group in question and by the position of the SH groups. For example, in dithiol 10 the COOH group is more electronegative than the OH group in dithiol 1; consequently the hydrogen atoms of the thiol groups may possibly be drawn closer to the molecule, and specifically to the sulphur atom. Therefore, the degree of dissociation of the SH groups in dithiol 10 would be less, compared to that of dithiol 1, and the rate of oxidation slower. Similarly the ester derivatives of dithiol 10, being less electronegative than the acid, should be oxidized at a faster rate. This has been found to be the case. Again, the ether derivatives of dithiol 1, being less electronegative than the acid, have a faster rate of oxidation than dithiol 10. The group C_6H_5 is also a strong electronegative group. The two phenyl derivatives (dithiols 7 and 17) were oxidized at a much slower rate than dithiol 1. It is also apparent that the longer the chain of carbon atoms, the slower the rate of oxidation of the molecule. For example, among the esters the relative rates of oxidation were as follows: methyl (dithiol 11) > ethyl (dithiol 8) > *n*-propyl (dithiol 18) > *n*-butyl (dithiol 19). For the ethers, the methyl ether derivative (dithiol 13) > ethyl (dithiol 9) > *iso*propyl (dithiol 20).

The distance of the SH groups from each other has also an effect on the rate of oxidation. Separation of the SH groups decreased the rate of oxidation so that when they were separated from each other by four C atoms there was practically no oxidation. This was also the case of dithiol 6, where the SH groups are attached at the 1:5 positions of the naphthalene ring. This effect may be explained by assuming that oxidation is preceded by the intermediate formation of the unstable copper cyclic compound

If the rate-determining step in the oxidation process is the formation of this unstable cyclic complex it might help us to understand why the introduction of a CH_2 group between the two thiols (dithiol 3) prevents catalysis by copper. It would also explain the relative ease of oxidation of dithiol 2 and the stability of the trithiol 5. In this case, the two outer SH groups may be competing for the middle SH group to form the cyclic complex. The existence of such unstable metal-thiol complexes during the oxidation of thiols has been observed in the oxidation of cysteine by iron (Michaelis & Barron, 1929).

SUMMARY

1. Dithiols are sluggish oxidation-reduction systems, non-autoxidizable, and rapidly oxidized in the presence of a number of catalysts. Copper and iron-protoporphyrin are the most powerful oxidation catalysts. $FeCl_3$, ferricyanide and cytochrome oxidase (in the presence of cytochrome *c*) also oxidize dithiols.

2. Oxidation by ferricyanide can be used for the estimation of dithiols, either manometrically or colorimetrically.

3. Dithiols combine with a number of heavy metals, forming complex compounds, mostly insoluble. Fe, Pb, Sn, Bi, Cu, Co, Ni, Se and Sb form coloured complexes. Zn, Cd and Hg give white precipitates. Mg combines, forming colourless soluble compounds.

4. In the presence of oxygen dithiols destroy iron-porphyrin compounds (haemin, oxyhaemoglobin) by opening the porphyrin ring. There is disappearance of the Soret band. Dithiols react instantaneously with methaemoglobin and reduce it to haemoglobin.

5. The rate of oxidation of dithiols with copper as catalyst seems to depend on the pH of the solution, on the distance of the SH groups from each other and on the electronegativity of the residual groups.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

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Reactivation by Dithiols of Enzymes Inhibited by Lewisite

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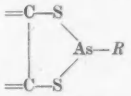
The protein component of a number of enzyme systems contains SH groups which are either freely reacting or sluggish. The freely reacting SH groups give the nitroprusside test in the native protein, are easily oxidized by mild oxidizing agents and alkylating agents in low concentrations (iodoacetate, mustard gas, diphosgene, cyanogen chloride), and readily form mercaptides. On denaturation, the sluggish SH groups become freely acting. Sulphydryl enzymes are widely distributed, existing among the enzymes concerned with the metabolism of proteins, fats and carbohydrates. Since the metabolism of foodstuffs is performed by a series of enzymic reactions which in some steps are linked to each other, it follows that inhibition of sulphydryl enzymes will create profound disturbances in the metabolism of the body. Inhibition of any of these SH enzymes by oxidizing agents or mercaptide-forming agents can be counteracted by addition of thiol compounds. In fact, thiols were used successfully years ago by Voegtlin, Dyer & Leonard (1923), Voegtlin, Rosenthal & Johnson (1931), and by Eagle (1939), in the treatment of arsenical poisoning in animals. Monothiols, however, were often found inefficacious. The introduction of dithiols (Peters, Stocken & Thompson, 1945) has marked a great progress in the reactivation of SH enzymes inhibited by oxidizing or mercaptide-forming agents, and hence in the treatment of intoxications produced by these substances.

This paper is concerned with the inhibition of SH enzymes by lewisite and their reactivation by dithiols.

EXPERIMENTAL AND RESULTS

Inhibitions produced by arsenic and subsequent reactivation

It is well known that trivalent arsenic combines with thiol compounds giving thioarsinites which are soluble and can be dissociated quite easily (Cohen, King & Strangeways, 1931). If the arsenical combines with the SH groups of sulphydryl enzymes, this combination may be broken by addition of thiol compounds. Stocken & Thompson (1941) postulated on the basis of their studies on kerateine solutions treated with lewisite that the thioarsinites thus formed had the configuration:



Bartlett, Cohen, Dauben, Rosen & Ryan (personal communication) confirmed the cyclic nature of the dithiol compounds with lewisite. It was thought then that dissociation of the thioarsinites formed with the SH groups of the protein molecule could be more easily performed by thiols possessing the property of forming more stable cyclic compounds. In fact, Stocken & Thompson (1941) found that brain pyruvate oxidase inhibited by lewisite could be reactivated by 2:3-dimercaptopropanol (BAL), while equal concentrations of cysteine had no effect at all.

Succinoxidase is a sulphydryl enzyme (Hopkins & Morgan, 1938). It was inhibited 96% by 5×10^{-5} M-lewisite and was completely reactivated on addition of $2{\cdot}5 \times 10^{-4}$ M-BAL. On addition of $1{\cdot}25 \times 10^{-4}$ M-dithiol there was 83% reactivation. (To produce this reactivation, 2·5 mol. of dithiol were thus necessary against 1 mol. of lewisite.) Addition of 5×10^{-4} M-glutathione (GSH) (equivalent in SH groups to $2{\cdot}5 \times 10^{-4}$ M-dithiol) produced only 23% reactivation. Liver choline oxidase was inhibited 74% by 5×10^{-4} M-lewisite; on addition of $1{\cdot}25 \times 10^{-3}$ M-dithiol there was 37% inhibition, i.e. the enzyme was reactivated 37%. This partial reactivation is probably due to rapid oxidation of dithiol by the ground liver preparation. The oxidation of ethanol by yeast alcohol oxidase was 40% inhibited on addition of $2{\cdot}5 \times 10^{-4}$ M-lewisite; 1×10^{-3} M-dithiol produced 20% reactivation (Table 1).

In Table 2 is given a list of the enzyme systems which were tested with lewisite. It can be seen that not all SH enzymes were inhibited by lewisite, striking examples being yeast carboxylase and *d*-amino-acid oxidase; both, however, are inhibited by *p*-chloromercuric benzoic acid and reactivated by glutathione (Barron & Singer, 1945; Singer & Barron, 1945).

Table 1. *Reactivation by thiols of enzymes inhibited by lewisite*

Enzyme	Lewisite (M)	Inhibition (%)	GSH Molarity	GSH Reactivation (%)	BAL Molarity	BAL Reactivation (%)
Succinoxidase	5×10^{-5}	96	5×10^{-4}	23	$2{\cdot}5 \times 10^{-4}$	Complete
Choline oxidase	5×10^{-4}	74	—	—	$1{\cdot}25 \times 10^{-3}$	37
Alcohol oxidase (yeast)	$2{\cdot}5 \times 10^{-4}$	40	—	—	1×10^{-3}	20

Table 2. *Effect of lewisite on the activity of isolated enzyme systems*

Enzyme system	Lewisite concentration (M)	Substrates	Determination	Inhibition (%)
Arginase (liver)	1×10^{-4}	Arginine	Urea formation	None
	1×10^{-3}	Arginine	Urea formation	87
Alcohol oxidase, yeast (activating protein)	5×10^{-4}	Ethanol	Diphosphopyridine nucleotide reduction	60
Alcohol oxidase, liver (activating protein)	1×10^{-3}	Ethanol	Diphosphopyridine nucleotide reduction	None
Lactic oxidase, liver (activating protein)	1×10^{-3}	Lactate	Diphosphopyridine nucleotide reduction	None
Uricase	1×10^{-3}	Uric acid	O_2 uptake	None
Pepsin	1×10	Haemoglobin	Tyrosine formation	None
Trypsin	1×10	Haemoglobin	Tyrosine formation	None
Choline oxidase, liver	5×10^{-4}	Choline	O_2 uptake	72
Choline esterase, serum	1×10^{-4}	Acetylcholine	Acetylcholine hydrolysis	85
Cytochrome oxidase, muscle	1×10^{-3}	Hydroquinone	O_2 uptake	None
Succinoxidase, muscle (activating protein)	5×10^{-5}	Succinate	O_2 uptake	96
Catalase	1×10^{-3}	H_2O_2	H_2O_2 left undestroyed	None
Transaminase, muscle	1×10^{-3}	Pyruvate + glutamate	Pyruvate disappearance	33
Diamine oxidase, kidney	1×10^{-3}	Histamine	O_2 uptake	None
Carboxylase, yeast	1×10^{-3}	Pyruvate	CO_2 production	None
Urease	1×10^{-3}	Urea	CO_2 production	None
Citric dehydrogenase, liver	1×10^{-3}	Citrate	Cresyl blue reduction	None
Pyruvate oxidase (gonococci)	1×10^{-4}	Pyruvate	O_2 uptake	Complete
Starch phosphorylase	1×10^{-3}	Hexose-1-phosphate	Inorganic P formation	None
Acid phosphatase	1×10^{-3}	β-Glycero-phosphate	Inorganic P formation	24
Alkaline phosphatase	1×10^{-3}	β-Glycero-phosphate	Inorganic P formation	None
d-Amino-acid oxidase	1×10^{-4}	*dl*-Alanine	Pyruvate formation	None

Table 3. *Effect of lewisite on tissue respiration*

(Tissue slices; lewisite added after neutralization in water, final concentration 1×10^{-4} M. Buffer, Ringer-phosphate pH 7·4. Temp. 38°. Figures give μl./hr./mg. dry wt.)

Tissue	Substrate	O_2 uptake: Control (μl.)	O_2 uptake: Inhibitor (μl.)	Inhibition (%)
Kidney (rat)	None	18·47	6·62	64
Kidney (rat)	Succinate	27·8	11·05	60
Kidney (rat)*	None	20·6	0·61	97
Kidney (rat)	Pyruvate 1st hr.	30·0	4·2	86
Kidney (rat)	Pyruvate 2nd hr.	32·8	0·5	98·5
Liver (pigeon)	None	7·13	3·87	46
Liver (pigeon)	Pyruvate	8·17	3·99	51
Testis (rat)	Glucose 1st hr.	9·04	5·76	36
Testis (rat)	Glucose 2nd hr.	8·80	2·47	72
Spleen (rat)	Glucose 1st hr.	10·92	6·82	37·5
Spleen (rat)	Glucose 2nd hr.	10·89	3·39	69
Lung (rat)	Glucose 1st hr.	6·96	3·53	49
Lung (rat)	Glucose 2nd hr.	7·05	1·56	78
Brain (rat)	Pyruvate 1st hr.	12·98	1·80	86
Brain (rat)	Pyruvate 2nd hr.	12·08	0·25	98
Kidney (rat)	*dl*-Alanine 90 min.	42·05	12·7	70
Kidney (rat)	*l*(+)-glutamate 1st hr.	45·9	19·6	57
Kidney (rat)	*l*(+)-glutamate 2nd hr.	34·9	4·8	86
Skin (lewisite) applied to skin of living rat)	Glucose	0·47	0	Complete

* Inhibitor concentration 1×10^{-3} M.

Table 4. *The effect of arsenicals on the oxidation of succinate by succinic oxidase (from pigeon breas muscle), and on the respiration of brain slices (rat)*

Inhibitor	Concentration (M)	O_2 uptake: Control (μl.)	O_2 uptake: Inhibitor (μl.)	Inhibition (%)
Succinic oxidase:				
Arsenite	5×10^{-3}	232·5	197	15
p-Carboxyphenyl arsine oxide (I)	5×10^{-5}	217·5	5·3	63
Lewisite	5×10^{-5}	236	10	96
Brain:				
Arsenite	1×10^{-4}	13·9	10·3	26
(I)	1×10^{-4}	13·9	12·5	9·5
Lewisite	1×10^{-4}	13·9	4·6	66·6
Kidney, 1st hr.:				
Arsenite	1×10^{-4}	45·9	33·2	27·6
(I)	1×10^{-4}	45·9	38·3	16·5
Lewisite	1×10^{-4}	45·9	19·6	57·4
Kidney, 2nd hr.:				
Arsenite	1×10^{-4}	34·9	21·9	37
(I)	1×10^{-4}	34·9	20·7	40
Lewisite	1×10^{-4}	34·9	4·8	86

Lewisite previously hydrolyzed in water and neutralized to pH 7·4 inhibited to a great extent the respiration of tissue slices; the degree of inhibition in every case increased with time. Thus, for instance, the respiration of rat testis was inhibited 36 % in the first hour after addition of 1×10^{-4} M-lewisite and 72 % in the second hour. Lewisite applied to the skin of a living rat which was killed 2 hr. later inhibited the respiration of skin slices completely (Table 3).

A comparison of the inhibiting power of arsenite (arsenious oxide neutralized to pH 7·4), of organic arsenicals used in the treatment of syphilis, and lewisite shows that the divinyl group of lewisite greatly enhanced the inhibiting action of arsenic on tissue respiration (Table 4). The same results were obtained with succinoxidase. Arsenite (5×10^{-3} M) inhibited 15 %; *p*-carboxyphenylarsine oxide (5×10^{-5} M) inhibited 63 %; and lewisite (5×10^{-5} M) inhibited 96 %. In yeast alcohol dehydrogenase, arsenite at a concentration of 5×10^{-4} M produced only a slight inhibition; $2{\cdot}5 \times 10^{-4}$ M-*p*-carboxyphenylarsine oxide inhibited by 30 %, and $2{\cdot}5 \times 10^{-4}$ M-lewisite by 60 %. Addition of BAL reduced the inhibition to 40 % (Fig. 1).

With kidney slices and pyruvate as the oxidizable substrate, 5×10^{-5} M-lewisite produced 67 % inhibition on the O_2 uptake. On addition of BAL ($2{\cdot}5 \times 10^{-4}$ and $1{\cdot}25 \times 10^{-4}$ M) 20 min. after the addition of lewisite there was complete reactivation of the rate of respiration. On the other hand, the addition of $2{\cdot}5 \times 10^{-3}$ M-glutathione produced only 17 % reactivation, while 5×10^{-4} M had no effect at all (Table 5). Similar results were obtained when the utilization of pyruvate by kidney slices was measured.

Lewisite (5×10^{-5} M) inhibited the utilization of pyruvate by 88 %. This inhibition was completely released on addition of $2{\cdot}5 \times 10^{-4}$ M-BAL; a concentration of $1{\cdot}25 \times 10^{-4}$ M, i.e. 2·5 times the concentration of lewisite, released the inhibition by 94 %.

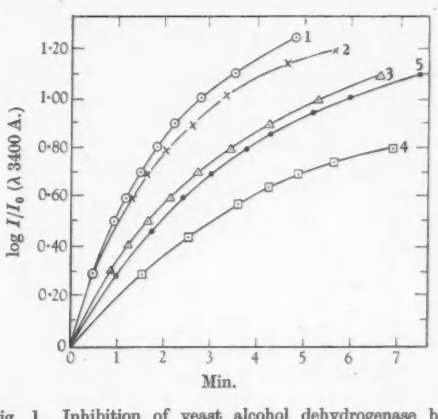


Fig. 1. Inhibition of yeast alcohol dehydrogenase by arsenicals, as measured by the rate of reduction of diphosphopyridine nucleotide (DPN). Temp. 25°, pH 7·7, DPN added at time 0.
1, control; 2, Na arsenite, 5×10^{-4} M; 3, *p*-carboxyphenylarsine oxide, $2{\cdot}5 \times 10^{-4}$ M; 4, lewisite, $2{\cdot}5 \times 10^{-4}$ M; 5, lewisite + BAL, 1×10^{-3} M.

Glutathione, however, produced only slight reactivation, a concentration of $2{\cdot}5 \times 10^{-3}$ M reactivating by 20 %. Similar results were obtained with brain tissue by Peters, Sinclair & Thompson (1946).

Table 5. *Effect of lewisite on the O_2 uptake and pyruvate utilization by the kidney: reactivation with glutathione and BAL added 15 min. after lewisite*

(Buffer, Ringer-phosphate, pH 7·4; pyruvate, 0·01 M; lewisite, 5×10^{-5} M; temp. 38°; O_2 uptake in μl./mg. dry wt./hr; pyruvate utilization, μmol./mg./90 min.)

Exp. conditions	Reactivator conc. (M)	O_2 uptake	Inhibition (%)	Pyruvate utilization	Inhibition (%)
Control	—	28·3	—	2·28	—
Lewisite	—	9·3	67	0·28	88
Lewisite + BAL	$2·5 \times 10^{-4}$	31·5	None	2·63	None
Lewisite + BAL	$1·25 \times 10^{-4}$	32·9	None	2·15	6
Lewisite + GSH	$2·5 \times 10^{-3}$	14·2	50	0·47	79·5
Lewisite + GSH	5×10^{-4}	8·6	70	0·35	84·5

Table 6. *Inhibition by lewisite of the oxidation of α-ketoglutarate by kidney slices*

(Lewisite, 1×10^{-4} M; reactivation by BAL ($2·5 \times 10^{-3}$ M) and glutathione (5×10^{-3} M); O_2 uptake in μl./mg. dry wt./hr.)

			Ketoglutarate	
Inhibitor	O_2 uptake	Inhibition (%)	Utilization (%)	Inhibition (%)
Control	21	—	98	—
Lewisite	5·9	72	14	87
Lewisite + glutathione	13·7	34	52	47
Lewisite + BAL	19·5	6	83	15·5

The oxidation of α-ketoglutarate by kidney slices, as determined by the oxygen uptake and the utilization of α-ketoglutarate, was inhibited by lewisite (1×10^{-4} M). The inhibition of the O_2 uptake was 72 %; the inhibition of α-ketoglutarate utilization, 87 %. BAL (5×10^{-3} M) produced a reactivation on the O_2 uptake of 66 %, and of 72 % on the utilization of α-ketoglutarate. Glutathione at the same concentration was less effective (38 % reactivation of the O_2 uptake and 40 % of α-ketoglutarate utilization) (Table 6).

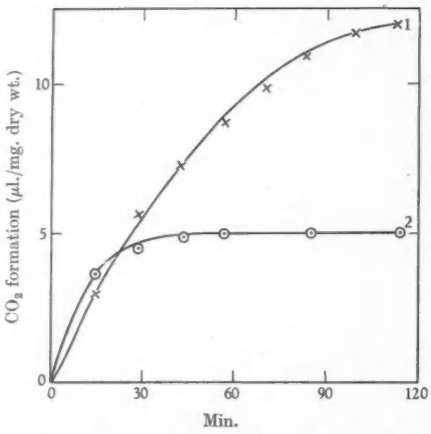


Fig. 2. Effect of lewisite on brain glycolysis when added immediately before measurement of the rate of glycolysis (CO_2 formation). Buffer, Ringer-bicarbonate, pH 7·4; N_2-CO_2 as gas phase. 1, control; 2, effect of 1×10^{-4} M-lewisite.

When lewisite (neutralized) was added immediately before measurement of the rate of glycolysis in brain slices (CO_2 production in Ringer-bicarbonate buffer) inhibition did not start until 15 min. later, reaching its maximum at the end of 45 min. (Fig. 2). The inhibition produced on addition of 1×10^{-5} M-lewisite was completely released by $2·5 \times 10^{-4}$ M-BAL. Glutathione, on the other hand, prevented the inhibition when added before the addition of lewisite; it produced a reactivation of 37 % when added 10 min. later, and no effect at all when added 30 min. later (Table 7).

The synthesis of carbohydrate from pyruvate by rat kidney slices was completely inhibited with 1×10^{-4} M-lewisite; 1×10^{-5} M inhibited by 50 %, and 5×10^{-6} M by 15 %. There are many steps in the series of enzymic reactions from pyruvate to carbohydrate where the protein moiety contains SH groups susceptible to inhibition by lewisite. The synthesis of acetoacetate from pyruvate by minced pigeon liver in the presence of malonate was inhibited by lewisite (Table 8).

DISCUSSION

Of the numerous arsenicals used for the inhibition of SH enzymes, lewisite was usually the most effective as shown in experiments with succinoxidase, yeast alcohol dehydrogenase and in experiments where the respiration of tissue slices was measured; the organic trivalent arsenicals came next in inhibitory power; and the arsenites were least effective. This greater affinity of lewisite for the SH groups of

Table 7. *Inhibition of the anaerobic glycolysis of brain slices (rat) by lewisite and reactivation with glutathione and BAL*

(Buffer, Ringer-bicarbonate; gas phase, N_2:CO_2; pH 7·4; temp. 38°; glucose concentration, 0·01 M; lewisite, 1×10^{-4} M.)

Exp. conditions	Reactivation concentration (M)	$Q^{N_2}_{CO_2}$ (μl.)	Inhibition (%)
Control	—	7·78	—
Lewisite	—	0·82	89
Lewisite + BAL	5×10^{-4}	10·14	None
Lewisite + BAL	$2{\cdot}5 \times 10^{-4}$	11·68	None
Lewisite + GSH added 10 min. later	5×10^{-3}	3·76	51·6
Lewisite + GSH added 30 min. later	5×10^{-3}	1·59	80
GSH before lewisite	5×10^{-4}	7·80	None

Table 8. *Effect of lewisite on the synthesis of carbohydrate by kidney slices (rat) and the synthesis of acetoacetate by minced pigeon liver*

Carbohydrate synthesis: Buffer, Ringer-$NaHCO_3$; gas phase, O_2:CO_2; pH 7·4; pyruvate concentration, 0·02 M; temp. 38°; incubation time, 3 hr.

Acetoacetate synthesis: Minced pigeon liver suspended in 0·121 M-KCl + 0·02 M-phosphate, pH 7·4; 0·02 M-pyruvate + 0·02 M-malonate. (1 μmol. acetoacetate = 22,400 μl.)

	Carbohydrate synthesis		Acetoacetate synthesis	
Lewisite conc. (M)	(μmol. glucose/ 100 mg. tissue)	Inhibition (%)	(μl./hr.)	Inhibition
None	3·62	—	274	—
1×10^{-4}	0·35	97	—	—
1×10^{-5}	1·18	50	—	—
5×10^{-6}	3·08	15	—	—
5×10^{-4}	—	—	0	Complete

enzymes must be due to the presence of the vinyl groups in the molecule, for the lewisite used in all the experiments was previously hydrolyzed and made neutral, existing presumably as Cl.CH:CH.AsO. In general, lewisite combines rapidly with the freely reacting SH groups while it has no action on the sluggish SH groups of the protein molecule. There were, however, three notable exceptions: *d*-amino-acid oxidase and yeast carboxylase which are reversibly inhibited by *p*-aminophenyldichloroarsine-HCl (Barron & Singer, 1945) were not inhibited by lewisite; and transaminase, which was largely inhibited by *p*-carboxyphenylarsine oxide (81% inhibition), was only 33% with the same concentration of lewisite. Possibly these differences are due to the spatial distribution of the SH groups in the protein molecule. Among the inhibitions produced by lewisite, that of carbohydrate synthesis from pyruvate is extremely interesting, not only because it is produced at very low concentrations but also because it may help us to understand the mechanism of synthesis. Of all the reactions where pyruvate takes part and where SH enzymes are required (oxidation, dismutation, carboxylation, decarboxylation, transamination) it is the oxidative reaction which is the most sensitive to the inhibiting action of lewisite as was demonstrated for the first time by Peters *et al.* (1940, 1946). Since the amounts of lewisite required to produce complete inhibition of carbohydrate synthesis have only a partial effect in all pathways of pyruvate metabolism except the oxidative pathway, it is reasonable to postulate that the synthesis of carbohydrate from pyruvate starts with the formation of an active acetyl group produced by the oxidation of pyruvate. Synthesis would proceed via condensation of this acetyl group. The great superiority of BAL on producing reactivations of enzyme inhibition by lewisite shown by the Oxford workers has been confirmed and extended in this paper.

SUMMARY

1. Among the trivalent arsenical compounds (organic arsenicals, arsenite, lewisite) able to form reversible thioarsinites, lewisite has in general the greatest affinity for the SH groups of the protein moiety of enzymes requiring those groups for activity. It inhibited all the sulphydryl enzymes with the exception of *d*-amino-acid oxidase, yeast carboxylase, and transaminase. In every case enzyme inhibition was reversed by the addition of BAL, which was found to be a better reversal agent than glutathione.

2. Lewisite inhibited the respiration of tissue slices, the inhibition being reversed on addition of BAL and to a lesser degree on addition of glutathione. It inhibited anaerobic glycolysis, this inhibition

being reversed completely by BAL and only partially by glutathione. Lewisite inhibited also the synthesis of carbohydrate from pyruvate by kidney slices.

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* Information available on application to Ministry of Supply, London.

The Action of British Anti-lewisite (BAL) on Enzyme Systems*

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It has been shown by Peters and others that 2:3-dimercaptopropanol (BAL) can be used as an antidote to lewisite, and can prevent vesication of human skin by lewisite if applied within 1 hr. after contamination. It was also shown not only to protect the arsenic-sensitive pyruvic oxidase from the inhibitory action of the arsenical *in vitro*, but to reverse poisoning which had already occurred, owing to its high affinity for arsenic. The work on BAL has been briefly reviewed by Peters, Stocken & Thompson (1945). BAL is itself toxic in large doses, with an LD_{50} of 110 mg./kg. by subcutaneous injection in rats. In an attempt to study the cause of this toxicity the action of BAL on a large number of enzymes was investigated *in vitro*.

EXPERIMENTAL

Methods. For each enzyme the conditions of estimation (amount of enzyme, etc.) were so arranged that the enzyme concentration was limiting, i.e. such that the rate of reaction was proportional to the amount of active enzyme present.

A pure specimen of BAL, obtained from Prof. Peters, was used throughout this work as a stock M/4 solution in ethylene glycol monoethyl ether. The required concentration of BAL was added to the enzyme solution in buffer and incubated with it for 15 min. before starting the reaction by addition of the substrate. Zymohexase and creatine phosphokinase† were tested in veronal buffer at pH 7·4, pyrophosphatase in veronal buffer at pH 7·9, and *d*-amino-acid oxidase in phosphate buffer at pH 7·7. All other experiments were carried out at pH 7·4 in phosphate or bicarbonate buffer.

Enzymes. These were prepared from the following materials: polyphenol oxidase from mushrooms (Keilin & Mann, 1938); carbonic anhydrase from mammalian erythrocytes (Keilin & Mann, 1940); catalase from ox liver (Sumner & Dounce, 1939); peroxidase from cow's milk (Elliott, 1932); aldehyde mutase from ox liver (Dixon & Lutwak-Mann, 1937); phosphorylase from potato (Hanes, 1940); xanthine oxidase from milk (Dixon & Thurlow, 1924); *d*-amino-acid oxidase from ox kidney (Krebs, 1935); cholinesterase from horse serum (Stedman & Stedman, 1935) and from rabbit brain (a strained tissue-suspension in bicarbonate-Ringer); glyoxalase from ox liver (a dialyzed extract of acetone-dried tissue); pyro-

* This work formed a part of the research programme carried out for the Ministry of Supply by an Extra-Mural Research team under the direction of Dr M. Dixon, and was reported to the Ministry in 1943 (Webb & van Heyningen, 1943).

† The term phosphokinase has been suggested by Needham & Dixon (1942) as a general name for enzymes of the hexokinase group which catalyze reactions of the type

$$X + \text{Adenosinetriphosphate} \rightleftharpoons X\text{-phosphate} + \text{Adenosinediphosphate}.$$

phosphatase from rabbit kidney (an extract in 0·5 M-KCl, dialyzed); hexokinase from baker's yeast (van Heyningen, unpublished). Pepsin, trypsin and papain were commercial preparations (British Drug Houses Ltd.); creatine phosphokinase, zymohexase and the glycolysis system were studied in a dialyzed extract from an acetone precipitate of rabbit muscle extract; and cytochrome oxidase and succinic dehydrogenase were studied in an exhaustively washed ground pig-heart suspension (Keilin & Hartree, 1938).

RESULTS

The inhibitory action of various concentrations of BAL on the enzymes listed above is shown in Table 1. The maximum concentration of BAL used was 0·01 M. Seven enzymes were strongly inhibited: polyphenol oxidase, carbonic anhydrase, catalase, peroxidase, aldehyde mutase, phosphorylase and

Table 1. *Inhibition of various enzymes by 2:3-dimercaptopropanol (BAL)*

(Incubated with BAL for 15 min. before addition of substrate.)

Enzyme	Substrate	Method of estimation	Temp. of incubation (°)	BAL (M)	Inhibition (%)
Polyphenol oxidase	Catechol	Oxygen uptake	17	0·0025	95–100
			17	0·001	95
Carbonic anhydrase	$NaHCO_3$-Na_2CO_3	CO_2 production	17	0·005	75
			17	0·0025	60
			17	0·00125	50
Catalase	H_2O_2	O_2 production	17	0·0044	90
			17	0·0022	85
			17	0·0008	45
Peroxidase	(*a*) H_2O_2 and excess $NaNO_2$	Excess H_2O_2 estimated by catalase	17	0·0025	70
	(*b*) Pyrogallol and H_2O_2	Purpurogallin formamation	17	0·00125	60
Cytochrome oxidase	Cytochrome *c* and catechol	Oxygen uptake	37	0·005	0
Aldehyde mutase*	Acetaldehyde	CO_2 production	37	0·0042	90
Phosphorylase	Cori ester	Formation of inorganic phosphate	25	0·0025	40
Glyoxalase*	Methylglyoxal	CO_2 production	37	0·0028	80
Hexokinase*	Glucose and adenosinetriphosphate	CO_2 production in $NaHCO_3$ buffer	37	0·01	0 (*a*)
			37	0·01	40 (*b*)
Pyrophosphatase	$Na_4P_2O_7$	Formation of inorganic phosphate	25	0·008	0
Succinic dehydrogenase	Succinate	Oxygen uptake (with methylene blue as carrier)	37	0·00125	0
Xanthine oxidase	Hypoxanthine	Oxygen uptake	37	0·005	0
d-Amino-acid oxidase	*dl*-Alanine	Oxygen uptake	37	0·0025	0
Creatine phosphokinase	Creatine and adenosinetriphosphate	Formation of creatine phosphate	37	0·0125	0
Cholinesterase (serum)	Acetylcholine	CO_2 production	20	0·005	0
Cholinesterase (brain)	Acetylcholine	CO_2 production	37	0·0042	0
Pepsin	Casein	Formol titration	37	0·0042	0
Trypsin	Casein	Formol titration	37	0·0042	0
Papain	Casein	Formol titration	Some activation by BAL, the amount depending on previous treatment of the enzyme		
Zymohexase	Hexosediphosphate	Formation of alkali-labile phosphate	37	0·0025	Activated 20%
Glycolysis system	Cori ester	Acid production	37	0·005	Activated 60%

* *Aldehyde mutase*: excess coenzyme I was added with the substrate. *Glyoxalase*: 0·004 M-glutathione was added as activator, and the same result was obtained whether this was added with the substrate or with the BAL. *Hexokinase*: (*a*) 0·0033 M-$MgCl_2$ added with the enzyme; (*b*) no $MgCl_2$ added. All oxygen uptakes and gas outputs were measured in Barcroft manometers.

glyoxalase. Some of these effects were studied in more detail.

Polyphenol oxidase. This enzyme is almost completely inhibited by concentrations of BAL as low as 0·001 M. In an experiment designed to test whether this is due to combination with the copper prosthetic group (Keilin & Mann, 1938), the enzyme was incubated with M/400 BAL for 15 min. at 17°, and then $CuSO_4$ was added to give a final concentration of 0·01 M and the incubation continued for a further 15 min. The result, illustrated in Fig. 1, shows that the activity was almost completely restored by addition of Cu^{++}.

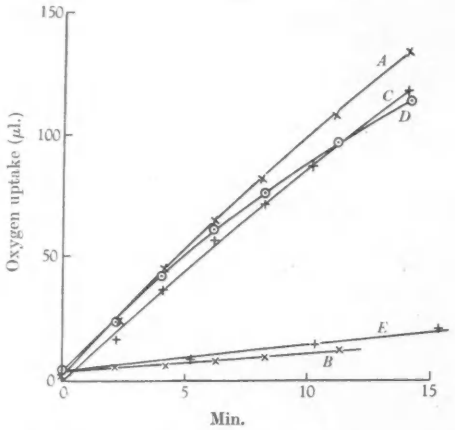

Fig. 1. Inhibition of polyphenol oxidase by BAL, and its reversal by Cu^{++}. Temp. 17°; pH 7·4. Reaction started by tipping in 6 mg. catechol. *A*, enzyme only (control). *B*, enzyme incubated with M/400 BAL for 30 min. *C*, enzyme incubated with M/400 BAL for 15 min., then for a further 15 min. after addition of M/100 $CuSO_4$. *D*, enzyme incubated in M/100 $CuSO_4$ for 15 min. *E*, no enzyme; oxidation in M/100 $CuSO_4$ only (blank for *C* and *D*).

Hexokinase. In the standard conditions for estimation of hexokinase worked out by van Heyningen (1942), $MgCl_2$ is added to give a concentration of M/300. Under these conditions BAL does not inhibit hexokinase at a concentration of 0·01 M. When no Mg^{++} is added, the hexokinase gives an activity about 17 % of the maximum, presumably due to traces of Mg^{++} in the enzyme preparation (an undialyzed solution of an ethanolic precipitate of autolyzed baker's yeast). This low activity was decreased by 40 % after incubation with 0·01 M-BAL. This is not due to an action on the enzyme itself, for if excess $MgCl_2$ is added after 20 min., the activity of the treated enzyme becomes equal to that of the control (Fig. 2).

The cytochrome system and oxidation of BAL. In dilute aqueous solution BAL undergoes atmospheric oxidation, each molecule taking up one atom of oxygen. The time for half-oxidation of an 0·005 M-solution in phosphate buffer pH 7·4, vigorously shaken in air, was about 70 min. at 17° and 20 min. at 37° (Fig. 3).

A solution of cytochrome *c* was rapidly reduced on addition of a few drops of BAL solution, the resulting liquid showing the normal absorption spectrum of reduced cytochrome *c*. It was not autoxidizable but on addition of a cytochrome oxidase preparation an absorption spectrum appeared which

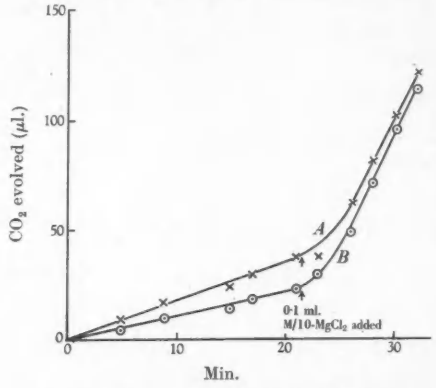

Fig. 2. Action of BAL on hexokinase: with and without addition of Mg^{++}. Temp. 38°; bicarbonate buffer pH 7·4. *A*, enzyme only (control); *B*, enzyme in M/400 BAL. Both incubated for 15 min. (in M/150 glucose for stabilization) before starting the reaction by addition of M/8-glucose and adenosinetriphosphate (1·5 mg. '7 min.' P). After 20 min., $MgCl_2$ was added to give a final concentration of M/300; the difference between the activity of the treated enzyme and the control disappeared.

was identical with that of the original oxidized cytochrome. Thus there was no evidence that BAL combined with cytochrome *c*, or interfered with its action as a hydrogen carrier.

In a manometric experiment, the amounts of cytochrome *c* and of cytochrome oxidase (a washed suspension of ground heart muscle) were such that the oxidase concentration was limiting. (This was investigated in a preliminary experiment with varying amounts of oxidase, the rate of oxygen uptake on addition of an excess of catechol being measured.) When BAL was tipped in, there was a rapid oxygen uptake, the rate remaining constant until almost one atom of oxygen/molecule of BAL had been taken up (Fig. 3, *A*). Since this initial rate was independent of the concentration of BAL remaining, the cytochrome system must have been

working at its maximum speed, i.e. the rate of oxygen uptake depended only on the active concentration of cytochrome oxidase. In another manometer containing the same amounts of cytochrome and oxidase, catechol was tipped from a Keilin tube. In this case the initial rate of oxidation was the same as that obtained with BAL (Fig. 3, *B*). Since catechol is not known to affect cytochrome oxidase, it was concluded that BAL likewise has no effect on the enzyme.

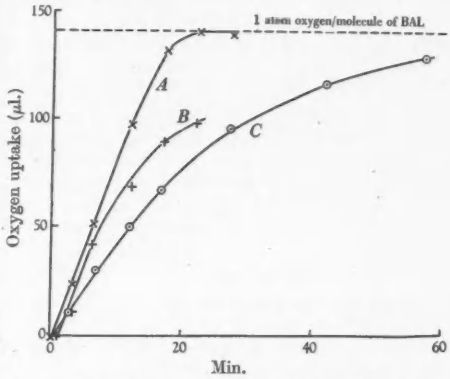

Fig. 3. Oxidation of BAL in M/200-aqueous solution. Temp. 37°; phosphate buffer pH 7·4. *A*, oxidation of 0·0125 mmol. of BAL by the cytochrome system, with limiting cytochrome oxidase. *B*, oxidation of 6 mg. catechol by cytochrome system similar to that in *A* (control for cytochrome oxidase activity). *C*, atmospheric oxidation of 0·0125 mmol. of BAL shaken in M/15 phosphate buffer.

Formation of BAL-metal complexes. The existence of stable compounds of BAL with copper, zinc, magnesium and iron was investigated qualitatively. On addition of excess $CuSO_4$ solution to a fresh 0·005 M-solution of BAL in water, a dark blue precipitate was produced. The precipitate was filtered off and the residual Cu^{++} estimated by titration with thiosulphate after addition of KI and H_2SO_4. The results showed the insoluble compound to contain one atom of Cu/mol. of BAL. The compound is a dark green powder, insoluble in water, HCl, ethanol, acetone or ether, and only slightly soluble in boiling 50 % HCl. It is much less readily oxidized than BAL and remains apparently unchanged after several months.

BAL (in 0·005 M-solution) gave a blue-green precipitate with ferric salts, but no precipitate with ferrous salts. (The ferric-BAL compound was soluble in strong ammonia to give a stable blood-red solution; addition of ammonia to an aqueous mixture of BAL and ferrous salt gave a similar result.) No insoluble compound of BAL with Zn^{++} or Mg^{++} was formed at neutral pH.

DISCUSSION

Of the seven enzymes inhibited by BAL, four are known to be metallo-proteins. Polyphenol oxidase contains Cu^{++}, carbonic anhydrase Zn^{++} (Keilin & Mann, 1940), and catalase and peroxidase an iron-containing prosthetic group (Keilin & Hartree, 1936; Elliott, 1932; Keilin & Mann, 1937). This strongly suggests that BAL specifically inhibits metal-enzymes and that this inhibition is due to a combination with the metals, for which BAL is known to have a high affinity. The only enzyme examined which is believed to be a metal enzyme and is not inhibited by BAL is cytochrome oxidase, which is probably part of the insoluble cytochrome complex (Keilin & Hartree, 1939). BAL is, however, rapidly oxidized by this enzyme. Cytochrome *c* itself is also unaffected.

Three enzymes other than the known metal enzymes were inhibited by BAL: glyoxalase, aldehyde mutase, and potato phosphorylase. Aldehyde mutase and phosphorylase have not been shown to be metal-containing enzymes but this possibility is perhaps not excluded. Glyoxalase needs glutathione as coenzyme (Lohmann, 1932), and in this case the inhibition may be competitive, the thiol grouping of the BAL having a higher affinity for the enzyme than has glutathione.

Papain, and the glycolytic system of muscle acetone powder, were both activated by BAL. Cysteine also activates these systems; the effect is probably due to the reducing properties of thiol groups. Zymohexase was also slightly activated; since this enzyme is known to be inhibited by traces of heavy metals (Herbert, Gordon, Subrahmanyan & Green, 1940), the apparent activation was probably due to the removal by BAL of traces of these metals in the undialyzed solution of muscle acetone powder used.

In the case of polyphenol oxidase, the effect of BAL is certainly due to formation of an insoluble copper-BAL compound, and the enzyme can be reactivated by the addition of excess copper salt. BAL probably acts as a general inhibitor of metal-containing enzymes and as an antagonist of metal activation of enzymes, even interfering with the activation of Mg^{++}-activated enzymes (e.g. hexokinase) by suboptimal concentrations of Mg^{++}. Whether the toxicity of the substance is due to this inhibition of metal-containing enzymes must remain an open question at present. BAL may be of value in future work for indicating whether an enzyme contains a metal prosthetic group or is dependent for its activity on traces of metal ions present in crude preparations.

SUMMARY

1. 2:3-Dimercaptopropanol (BAL) inhibits seven enzymes of a large number examined. Four of these,

polyphenol oxidase, carbonic anhydrase, catalase and peroxidase, are known to be metal-containing enzymes. The inhibition of glyoxalase is due to competition with glutathione, the activator of the enzyme. Aldehyde mutase and phosphorylase are also inhibited.

2. The inhibition of mushroom polyphenol oxidase by BAL is reversed by addition of excess of copper salt. This inhibition is due to the formation of an insoluble cupric-BAL compound.

3. The activity of hexokinase in the presence of suboptimal concentrations of Mg^{++} is diminished by BAL, an effect immediately reversed by addition of excess Mg^{++}.

4. BAL can be oxidized by the cytochrome system without any effect on the activity of the latter.

5. BAL may be considered as a potent inhibitor of metal-containing enzymes with the exception of the cytochrome system.

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* Information available on application to Ministry of Supply, London.

The Effect of 2:3-Dimercaptopropanol on the Activity of Enzymes and on the Metabolism of Tissues

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The brilliant work of Peters, and co-workers (see Peters, Stocken & Thompson, 1945), on the reversal of lewisite action by dithiol compounds which possess the property of forming stable cyclic arsenical rings has been one of the great achievements in the treatment of certain war-gas casualties. In fact, dithiols have already found a place among the useful drugs for the treatment of a number of intoxications (arsenicals or heavy metals). It was therefore necessary to study carefully the effect of dithiols on the activity of enzyme systems and on the metabolism of tissues in order to understand the toxic effects observed when dithiols in large amounts are injected into animals. Such a reducing agent, with rather negative oxidation-reduction potential, reacting readily with heavy metals, might produce enzyme inhibitions, either by maintaining in the reduced state the electron transfer catalysts of the oxidation enzyme systems, or by combining with the heavy metals which act as the prosthetic groups of the protein moiety of some enzymes. Webb & van Heyningen (1943) have, in fact, shown that 2:3-dimercaptopropanol can combine with the metals acting as prosthetic groups. The experiments presented in this paper demonstrate that the reducing power of dimercaptopropanol is also responsible for some of the toxic effects.

EXPERIMENTAL AND RESULTS

The effect of dimercaptopropanol on the activity of enzymes

Webb & van Heyningen (1943) have investigated the effect of dimercaptopropanol on a number of oxidative and hydrolytic enzymes. Enzymes possessing heavy metals as prosthetic groups were

inhibited (polyphenol oxidase with Cu, carbonic anhydrase with Zn, catalase and peroxidase with Fe^{+++} porphyrin as the prosthetic group; hexokinase and phosphorylase activated by Mg^{++}.) It was suggested that this inhibition might be due to combination of dithiol with the metal of the protein. In fact, inhibition of polyphenol oxidase was counteracted by addition of $CuSO_4$, and inhibition of hexokinase by addition of $MgCl_2$.

It has been stated by Webb & van Heyningen that dimercaptopropanol does not inhibit the system cytochrome-cytochrome oxidase. Since their experiments were performed by measuring manometrically the rate of oxidation of catechol and no precautions were taken to eliminate heavy metal contamination it was decided to reinvestigate this problem. (According to Webb & van Heyningen half-oxidation of 5×10^{-3} M-dimercaptopropanol at 38° occurred in 20 min. In this laboratory half-oxidation took place in 70 min.) The effect of dithiol on the rate of oxidation of cytochrome *c* was determined spectrophotometrically with cytochrome oxidase prepared according to Haas (1943).

Cytochrome *c* (2×10^{-5} M) was reduced with $Na_2S_2O_4$ and the excess of this reagent was oxidized by shaking the solution. To the solution containing varying concentrations

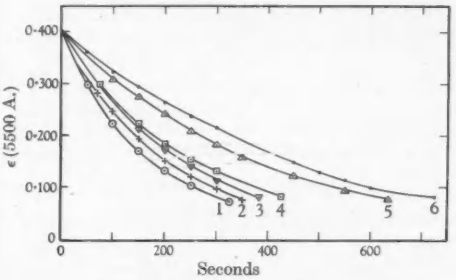

Fig. 1. Effect of BAL on the oxidation of reduced cytochrome *c* by cytochrome oxidase.

Curve no. ...	1	2	3	4	5	6
BAL conc. (M)	0	10^{-6}	5×10^{-6}	10^{-5}	5×10^{-5}	10^{-4}
Time of start of initial reaction (sec.)	9	8	26	106	630	1253

of dithiol plus reduced cytochrome *c* (all dissolved in 0·05 M-phosphate buffer, pH 7·1), there was added with a syringe 0·1 ml. of a dilute solution of cytochrome oxidase, and the rate of oxidation of reduced cytochrome *c* was followed. There occurred in the first place a delay in the initial oxidation of cytochrome *c*, as measured by the time required to diminish the extinction coefficient from the original value of 0·45 to 0·40. While in the absence of dithiol this initial oxidation time was 9 sec., in the presence of 5×10^{-6} M-dithiol it was 26 sec., with 1×10^{-5} M, it was 106 sec., 5×10^{-5} M 630 sec., and with 1×10^{-4} M (the M.L.D. for cats) it was delayed to 1253 sec. (Fig. 1). The rate of oxidation of reduced cytochrome *c* was also inhibited, the inhibition increasing as the concentration of dithiol increased.

The inhibition of cytochrome *c* oxidation must be attributed to the reducing power of dithiol, and is, therefore, quite different from the inhibition produced by HCN, for example, with cytochrome oxidase where there is no delay in the initial rate of oxidation.

According to Webb & van Heyningen, dithiol has no effect on the activity of serum-cholinesterase. The activity of this enzyme in the presence of dithiol was again determined with the purified enzyme from serum and with the enzyme from brain. The inhibition produced by 0·001 M-dithiol was small (Table 1). The other enzyme inhibitions reported by the above authors were also confirmed in this laboratory.

Webb & van Heyningen reported that dithiol had no effect on the activity of succinoxidase. Our experiments, as well as those of Rhoads (personal communication), show that dithiol can inhibit the activity of succinoxidase. When 2:3-dimercaptopropanol was added to pigeon-breast muscle succinoxidase immediately before succinate addition, there was slight inhibition (6·5 %); when dithiol was added to the enzyme 30 min. previous to succinate addition, i.e. when part of the dithiol was in the oxidized state, there was 25 % inhibition (Table 2). The inhibiting effect of oxidized dithiol can best be observed in rat-liver succinoxidase. This liver suspension contains an enzyme which rapidly oxidizes thiol compounds; here addition of dithiol produced complete inhibition of succinoxidase. Other factors responsible for the inhibition of succinoxidase by dithiols are the oxidation-reduction potential of the dithiol and the nature of the residual groups. As shown in Table 3, at a concentration of 1×10^{-3} M, few dithiols had low inhibitory power, these few being dithiols 2, 3 and 10 (the constitution of these and other numbered dithiols is given by Barron, Miller & Kalnitsky (1946)). Of these compounds with low inhibitory power (3, 4·5, 9·8 % inhibition respectively) dithiol 2 is oxidized twice as slowly as 2:3-dimercaptopropanol; dithiol 3 is very little oxidized; and dithiol 10, five times as slowly as dithiol 1. All the other compounds had a definite inhibitory effect. Dithiol 8 with a rate of oxidation about that of dithiol 1 produced half as much inhibition, the difference between these two being that dithiol 8 is an acid while dithiol 1 is an alcohol. The compound with the highest inhibitory power was dithiol 11, which has the fastest rate of oxidation. Of the three least inhibitory compounds (dithiols 2, 3 and 10) dithiols 2 and 10 are more toxic to animals than dithiol 1, while dithiol 3 has the same toxicity. Dithiol 11, the compound which produced the largest inhibition, is reported to be more effective than dithiol 1 in preventing the haemolysis

Table 1. *Effect of BAL on enzyme systems*

Enzyme	Source	Temp. (°)	Substrate	BAL (M)	Inhibition (%)	Reference
Polyphenol oxidase	Mushroom	17	Catechol	$2·5 \times 10^{-3}$	95–100	
Carbonic anhydrase	Red cells	17	HCO_3^-	$2·5 \times 10^{-3}$	60	Webb & van Heyningen (1943)
Catalase	Beef liver	17	H_2O_2	2×10^{-3}	85	
Peroxidase	Milk	17	H_2O_2-$NaNO_2$	$2·5 \times 10^{-3}$	60	
Cytochrome oxidase	Pig heart	37	Cytochrome *c*	5×10^{-3}	0	
Cytochrome oxidase	Pig heart	26	Cytochrome *c*	1×10^{-4}	49	Present authors
Aldehyde mutase	Beef liver	37	Acetaldehyde	4×10^{-3}	90	
Phosphorylase	Potato	25	Cori ester	$2·5 \times 10^{-3}$	40	Webb & van Heyningen (1943)
Glyoxalase	Beef liver	37	Methyl glyoxal	3×10^{-3}	80	
Hexokinase	Yeast	37	Glucose + ATP	1×10^{-2}	40	
Pyrophosphatase	Kidney	25	Na pyrophosphate	$2·5 \times 10^{-3}$	0	
Succinic oxidase	Heart	37	Na succinate	$1·25 \times 10^{-3}$	0	
Succinic oxidase	Pigeon breast	38	Na succinate	1×10^{-3}	65	Present authors
Xanthine oxidase	Milk	38	Hypoxanthine	5×10^{-3}	0	Webb & van Heyningen (1943)
d-Amino-acid oxidase	Kidney	38	*dl*-Alanine	$2·5 \times 10^{-3}$	0	
Creatine phosphokinase	Muscle	38	Creatine + ATP	$1·25 \times 10^{-2}$	0	
Cholinesterase (serum)	Blood serum	20	Acetylcholine	5×10^{-3}	0	
Cholinesterase (serum)	Blood serum	38	Acetylcholine	1×10^{-3}	16	Present authors
Cholinesterase (brain)	Rat	38	Acetylcholine	1×10^{-3}	7	Present authors
Pepsin		37	Casein	$4·16 \times 10^{-3}$	0	Webb & van Heyningen (1943)
Trypsin		37	Casein	$4·16 \times 10^{-3}$	0	
Papain		37	Casein	$4·16 \times 10^{-3}$	Some activation	
Lactic dehydrogenase	Heart	25	Lactate	1×10^{-3}	0	Present authors

Table 2. *Effect of BAL* (1×10^{-3} M) *on the activity of succinoxidase*

Experimental conditions	O_2 uptake (μl.)	Inhibition (%)
Enzyme from pigeon breast muscle		
A. Enzyme + BAL	0	—
Enzyme + succinate	292·5	—
BAL added before succinate	273·5	6·5
B. Enzyme + succinate	325	—
BAL + enzyme; succinate added 30 min. after BAL	243·8	25
Enzyme from rat liver		
Enzyme + BAL	60·8	—
Enzyme + succinate	311	—
Enzyme + succinate + BAL	41	Complete

Table 3. *Effect of dithiols* on the activity of succinoxidase*

(Each vessel contained 0·6 ml. enzyme; succinate, 0·03 M; 0·05 M-phosphate buffer, pH 7·0; dithiol, 0·001 M. Total vol. 3·0 ml; temp. 38°; duration of experiment, 30 min.)

Dithiol	Inhibition (%)	Dithiol	Inhibition (%)
1	25·0	12	77·0
2	3·0	13	84·6
3	4·5	14	64·6
4	73·0	15	82·5
5	58·3	16	30·4
6	41·0	17	63·0
7	79·5	18	23·5
8	30·1	19	69·6
9	85·4	20	89·6
10	9·8	21	59·5
11	95·3		

* For nomenclature of dithiols see Barron *et al.* (1946).

of red cells by arsine (Rhoads, personal communication).

Webb & van Heyningen reported that dithiol 1 did not inhibit the activity of *d*-amino-acid oxidase, an observation confirmed in this laboratory in studies with enzymes and with tissue slices. Neither the oxidation of *dl*-alanine, nor the oxidation of *l*-glutamate by kidney slices was affected by dithiol 1 (Table 4). The slight increase in the NH_3 formation

Table 4. *Effect of BAL* (10^{-3} M) *on utilization of amino-acids by kidney slices*

	Q_{O_2} 1st hr.	Q_{O_2} 2nd hr.	NH_3 formed (μmol./mg. dry wt. in 2 hr.)
No substrate	16·9	14·5	—
No substrate + BAL	8·4	5·2	—
dl-Alanine	28·6	24·1	0·82
dl-Alanine + BAL	28·9	24·1	1·30
l-Glutamic acid	33·5	33·3	0·66
l-Glutamic acid + BAL	28·0	27·2	0·67

in the presence of dithiol might be due either to reactivation of the enzyme (*d*-amino-acid oxidase is a SH enzyme) or to slight inhibition of NH_3 utilization by the tissues.

Krop (1946) has reported that 1×10^{-3} M-dithiol 1 produced an accumulation of lactate in isolated muscle; Durlacher, Bunting, Harrison, Ordway & Albrink (1946) have also reported an increase in the blood lactate concentration after injection of dithiol 1. The dithiol had no effect on the rate of reduction of diphosphopyridine nucleotide by lactate activated by lactic dehydrogenase.

Effect of 2:3-dimercaptopropanol on tissue metabolism

As reported by Webb & van Heyningen and by Rhoads, dimercaptopropanol inhibits the respiration of tissues. This inhibition increases with time and might be due to three factors: combination of dithiol with heavy metals necessary for enzyme activity; the delay produced by dithiol in the rate of oxidation of cytochrome *c* by cytochrome oxidase; and the inhibition of SH enzymes by oxidized dithiol.

The effect of dimercaptopropanol on the metabolism of pyruvate by tissue slices was not uniform. The oxidation of pyruvate by kidney slices (rat) was inhibited 22 % while the utilization of pyruvate by brain slices was unaffected (Table 5). The effect

Table 5. *Effect of BAL* (1×10^{-3} M) *on the* O_2 *uptake and untilization of pyruvate by rat tissues*

(Utilization of pyruvate is given as μmol./mg. dry wt. in 2 hr.)

	Q_{O_2} 1st hr.	Q_{O_2} 2nd hr.	Pyruvate utilized
Brain:			
No substrate	5·4	2·5	—
No substrate + BAL	5·2	0·88	—
Pyruvate	12·3	11·3	0·836
Pyruvate + BAL	11·4	7·4	0·782
Kidney:			
No substrate	22·1	18·1	—
No substrate + BAL	10·2	3·5	—
Pyruvate	30·3	31·9	2·30
Pyruvate + BAL	31·1	26·5	1·76
Diaphragm:			
No substrate	6·8	12·0	—
No substrate + BAL	3·4	5·5	—
Rectus Abdominalis:			
No substrate	3·0	6·0	—
No substrate + BAL	2·0	3·1	—

of dimercaptopropanol on the utilization of lactate by animal tissues was studied with the diaphragm, brain, and kidney of rats, the diaphragm having been cut into small strips, and the brain and kidney sliced. Diaphragm utilized 0·193 μmol. lactate/mg. dry wt. in 3 hr.; in the presence of dithiol there was no utilization of lactate. There was on the contrary an accumulation of 0·32 μmol./mg. tissue, which is in agreement with the findings of Krop. However, the utilization of lactate by brain and kidney was not altered in spite of the inhibition on the O_2 uptake observed after addition of dithiol (Table 6).

The extensive use of dimercaptopropanol in ointment form for percutaneous administration made necessary a study of its effects on the respiration of skin. BAL was applied to the skin of a 7-day-old rat; ½ hr. later the excess dithiol was wiped off with a gauze and the skin was cleaned with ethanol. The respiration was followed for 3 hr.; the Q_{O_2} values for the control skin and the treated skin were found to be practically identical. The effect of BAL on skin slices was also followed for 3 hr, 1×10^{-3} M-dithiol

Table 6. *Effect of BAL* (1×10^{-3} M) *on the utilization of lactate by tissues*

Tissue	Q_{O_2} 1st hr.	Q_{O_2} 2nd hr.	Lactate utilized (μmol./mg. dry wt. in 2 hr.)
Diaphragm:			
No substrate	5·39	4·61	—
No substrate + BAL	4·62	1·33	—
Lactate	6·45	6·63	0·193
Lactate + BAL	3·39	1·91	None
Brain:			
No substrate	4·83	2·20	—
No substrate + BAL	3·50	0·34	—
Lactate	8·30	6·26	0·54
Lactate + BAL	6·56	2·09	0·58
Kidney:			
No substrate	22·1	18·1	—
No substrate + BAL	10·2	3·5	—
Lactate	30·9	29·5	0·78
Lactate + BAL	25·3	20·1	0·67

being added to the Ringer-phosphate solution. Here, too, dithiol had no effect on the rate of respiration, for the increase observed in the first hour is probably due to the extra O_2 uptake from the oxidation of dithiol (Table 7). This resistance of skin respiration to BAL is remarkably in contrast with the inhibition observed in other tissues.

Table 7. *Effect of BAL on the respiration of young rat skin*

Experimental conditions	Q_{O_2} 1st hr.	Q_{O_2} 2nd hr.	Q_{O_2} 3rd hr.
BAL applied to skin of living rat; wiped off 30 min. later:			
Control	2·66	2·14	1·90
Treated	2·71	2·18	1·87
BAL added to skin slices in the Warburg vessel:			
Control	2·25	2·23	2·18
BAL	3·26	2·57	2·46

In the anaerobic glycolytic process in brain slices there are presumably the following metallo-proteins acting as components of enzyme systems: zymohexase, isomerase, triose mutase, enolase, and phosphopyruvate phosphatase. Since dithiols form complex compounds with a number of heavy metals, dithiol might inhibit glycolysis. In brain glycolysis there was, in the presence of 1×10^{-3} M-dimercaptopropanol a continuous inhibition which at the end of 2 hr. reached 47 %.

Dimercaptopropanol and insulin

It is known that the activity of insulin is destroyed by reduction of the S-S groups of the molecule. Since BAL has a strong reducing power ($E'_0 = -0{\cdot}150$ V. at pH 7·0), it might reduce these

Table 8. *The effect of BAL on insulin hypoglycaemia in rabbits*

Experimental conditions	Blood sugar in mg./100 ml. Insulin	BAL	BAL + insulin	Time after injection (min.)
1. BAL (0·01 M) + 8 units insulin in 30 ml. of 0·154 M-NaCl. 3 ml. injected/kg. wt. to fasting rabbits	111	107	115	Initial, fasting
	50	101	61	60
	44	98	42	120
	50	100	53	180
	56	99	52	240
	84	100	89	300
2. BAL (0·015 M) + 8 units insulin in 30 ml. of 0·154 M-NaCl. 3 ml. injected/kg. wt.	77	93	85	Initial, fasting
	53	87	67	60
	59	87	67	120
	87	91	96	180
	83	89	96	240
3. BAL (0·12 M) + 8 units insulin in 30 ml. of 0·154 M-NaCl. 3 ml. injected/kg. wt.	91	83	90	Initial, fasting
	51	59	114	60
	60	77	102	120
	95	Death	—	180
4. BAL (0·10 M) + 12 units insulin in 30 ml. of 0·04 M-phosphate, 0·1 M-NaCl. 3 ml. injected/kg. wt.	82	114	113	Initial, fasting
	39	103	123	60
	26	—	—	90
	36	84	104	120
	Death	87	106	180
	—	90	105	240

groups and thus destroy the effect of insulin. In fact, Durlacher *et al.* found an initial increase in blood sugar after the injection of dimercaptopropanol. To determine the effect of dimercaptopropanol on insulin the following experiments were performed.

Rabbits were injected intravenously with (*a*) BAL, (*b*) 0·8 unit of insulin/kg. wt., (*c*) 0·8 unit insulin plus BAL. In Exp. I (Table 8) 30 ml. of 0·01 M-BAL in 0·154 M-NaCl were kept for 5 min. at room temperature with 8 units of insulin (Iletin Lilly made from Zn-insulin). Dimercaptopropanol alone (3 ml. 0·01 M-dithiol/kg.) had no effect on the blood sugar; dithiol plus insulin lowered the blood sugar to the same extent as insulin alone. In Exp. 2, 30 ml. 0·015 M-dithiol were used and the contact of insulin with dithiol was increased to 10 min.; dithiol alone had no effect on the blood sugar; dithiol plus insulin produced less hypoglycaemia than insulin alone. In Exp. 3, 30 ml. 0·12 M-dithiol were used, the contact with insulin being 5 min. 3 ml. of this solution/kg. was fatal to the rabbits, the animals dying 3 hr. after the injection. Dithiol alone lowered the blood sugar level 60 min. after the injection, the blood sugar coming back to normal 120 min. later; dithiol plus insulin produced no hypoglycaemia. In Exp. 4, 40 ml. 0·1 M-phosphate, pH 7·4, plus 60 ml. 0·154 M-NaCl were boiled and saturated with N_2. To 30 ml. of this solution were added: (1) 12 units of insulin, (2) 12 units of insulin plus 0·2 ml. dithiol, (3) 0·2 ml. dithiol. Dithiol alone had no effect on the blood sugar; insulin alone produced marked hypoglycaemia.

SUMMARY

1. Dithiols produce inhibition of some enzyme systems by combining with the heavy metals that form the prosthetic group of the protein moiety of the enzyme. During the oxidation of dithiol there is inhibition of enzyme systems containing essential SH groups.

2. Dithiols reduce cytochrome *c* and thus show an apparent inhibition in the rate of oxidation of reduced cytochrome *c* by cytochrome oxidase. This inhibition is quite marked at concentrations which are lethal to animals.

3. Some dithiols produced a slight inhibition of succinoxidase, while others had a powerful inhibiting effect.

4. The reduced respiration of tissue slices brought about by dithiols is probably due to different factors: reduced rate of oxidation of cytochrome *c*; inhibition of metallo-protein enzymes; inhibition of SH enzymes by the oxidation product of dithiols.

5. 2:3-Dimercaptopropanol destroys the physiological activity of insulin. This destruction is probably due to the reduction of the S-S groups of insulin.

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The Electrophoretic Analysis of Protein Extracts from Striated Rabbit Muscle

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The electrophoresis method of Tiselius has been used only in a few instances for the study of muscle proteins. The purification of zymohexase has been followed by Bate Smith (1940), and Bailey (1942) has described experiments on myosin, which has been studied in more detail by Ziff & Moore (1944). Extracts of striated muscle from the rat and octopus have been examined electrophoretically by Mehl & Sexton (1942).

In an earlier paper (Dubuisson & Jacob, 1945*b*) the analysis of well-defined extracts from fatigued and resting frog striated muscle was described, but generally the characteristics of muscle proteins of mammals alone are relatively well established (Dubuisson, 1945). Further, electrophoretic examination reveals marked qualitative and quantitative differences between rabbit- and frog-muscle proteins. Consequently it was considered important to make a careful study of the muscle proteins of a mammal—the rabbit—with a view to defining accurately the characteristic properties of each of the main electrophoretic components.

METHODS

The electrophoresis apparatus used was that of Tiselius (1937), modified slightly (Dubuisson & Jacob, 1945*a*), the refractive index gradients being registered by the method of Longsworth (1939).

The preparation of muscle extracts. Rabbits were killed, bled and skinned, and immersed in ice-water for about 15 min. This causes an intense vasoconstriction which expels the blood almost quantitatively from the muscle, since no haemoglobin or plasma protein can be detected electrophoretically in the muscle extract (Jacob, 1945). The muscles were then rapidly dissected and ground in a cooled Latapie mincer. The pulp was mixed with 0·8 vol. of cold solvent and extracted with continuous mechanical stirring for an hour at 0°. The tissue debris was spun down for 5 min. at 6000 r.p.m. and 0°, the supernatant fluid removed and dialyzed in collodion tubes (8 cm. long, 1 cm. diam.) against a large volume of buffer (2 l./30 ml. extract). During the dialysis, of at least 24 hr. duration, the outer fluid was twice renewed and the collodion tube and diffusate were kept slowly moving. The last diffusate was used as supernatant fluid for the electrophoresis.

The extraction and dialysis fluids have been varied as required. In the first instance the results obtained using the following solutions will be described: extraction fluid 0·048 M-Na_2HPO_4–0·006 M-NaH_2PO_4, pH 7·70, μ=0·15; dialysis fluid Na_2HPO_4-NaH_2PO_4 (μ=0·1)+0·05 M-NaCl (total μ=0·15), pH varied from 5·38 to 8·04. An ionic strength of μ=0·15 allows relatively rapid electrophoresis without disturbing anomalies. For the extraction, pH 7·7 is optimal for all muscle proteins within the phosphate buffer range. Under these conditions the dialyzed extracts are deficient in the muscle proteins soluble only in phosphate buffers of higher ionic strength.

At all pH values a precipitate formed on dialysis which was removed both during and at the termination of dialysis by centrifuging. The quantity of precipitate varied considerably above pH 7·8, was least at pH 7·6, and became more abundant as the pH fell. The precipitates which formed above pH 7·1 were soluble in 0·5 M-KCl, and these solutions showed double refraction of flow.

In a second series of experiments the ionic strengths of the extraction fluid and of the dialyzing medium were varied, and the composition is specified for each individual case. The collodion tubes were not always completely impermeable to protein, but the amount of protein which occasionally passed through the membrane was very small. Cellophan tubes were not available. No antiseptics were used, but the normal precautions against denaturation were taken. Even at 0° the extracts were stable for a very short time, the stability decreasing with pH. Accordingly, the electrophoresis experiments were made within 2–5 days after extraction. If a longer time was allowed to elapse, changes occurred in the diagrams. The descriptions here are confined to normal diagrams.

RESULTS

I. Experiments at constant ionic strength

General description of diagrams

It is necessary first to summarize briefly the results already published (Jacob, 1945). At all pH values the electrophoretic diagrams show three groups of constituents (Figs. 1, 2). Group I, which accounts on the average for 72 % of the refractive increment of the extracts, is the most important, and consists of those proteins with the highest isoelectric points falling in the range pH 6·0–6·75. Group II, representing 16·8 % of the refractive increment, has isoelectric points in the region pH 5·0–5·5, while group III (5 % to 8·6 %) always has a considerable anodic mobility in phosphate buffers. The diagrams obtained between pH 7·4 and 8·0 (Fig. 1*a*, *b*) are the most complete. Group III separates first from the initial boundary after about 1 hr. electrophoresis at 4–5 V./cm., and consists of two peaks (*h* and *i*) which

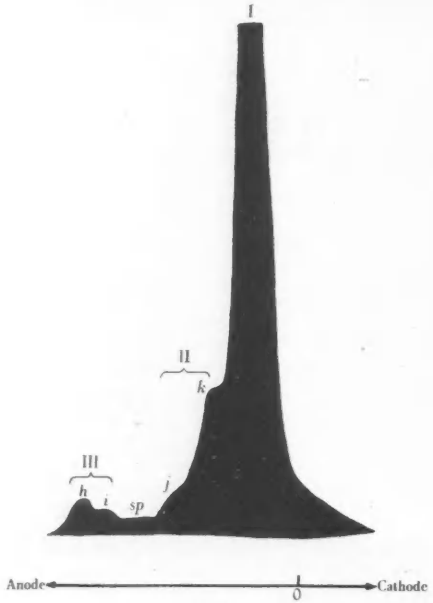

Fig. 1*a*.

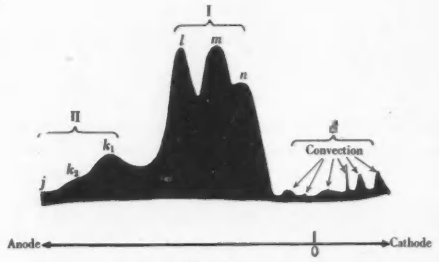

Fig. 1*b*.

Fig. 1. Electrophoretic patterns (anodic) of extracts from perfused skeletal muscles of rabbit. Exp. 38: ionic strength, 0·15; pH 7·52; electrical field, 4·58 V./cm.

Durations of electrophoresis: (*a*) 11,100 sec. (the boundary I is still unique and too sharp to be recorded completely); (*b*) 34,080 sec. (the faster moving boundaries have left the photographic field). Arrows indicate the direction of migration from the position of the initial boundary quoted as 0. Convection means convection disturbances (see p. 89).

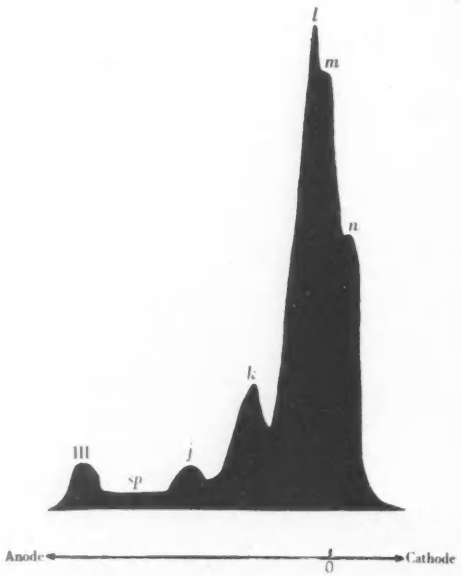

Fig. 2*a*

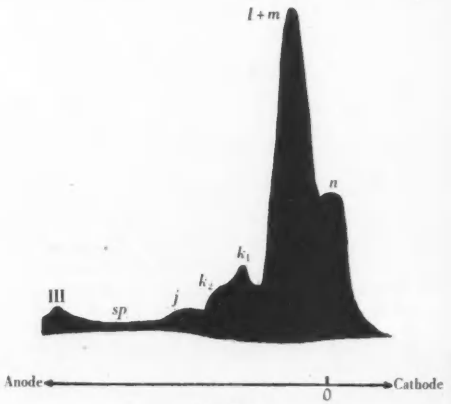

Fig. 2*b*.

Fig. 2. Modifications in the electrophoretic patterns related to pH changes at a constant ionic strength of 0·15. (*a*) Exp. 68 (anodic): pH 6·60; electrical field, 4·95 V./cm.; duration of electrophoresis, 13,800 sec. (*b*) Exp. 30: pH 6·30; electrical field, 4·79 V./cm.; duration of electrophoresis, 17,400 sec. (for this figure, the rate of motion of the schlieren diaphragm to that of the photographic plate is 1/14 instead of 1/20 as for the other patterns).

separate more completely from one another, the higher the pH. There may possibly be a peak faster than *h*, but owing to the small amount present and the wide spread of the peak, its estimation is outside the limits of accuracy of the method.

Group II separates after 2 hr. at 4–5 V./cm., and between it and group III is a broad band accounting for 2·6 % of the refractive increment. The upper border of this band (*sp*) is almost parallel to the base-line. Group II consists of two bands, *j* the faster and smaller, *k* the slower and larger. Peak *j* occasionally resolves into two peaks, and *k* usually resolves into two peaks of approximately equal area of which the slower, k_1, migrates with the initial velocity of *k*.

Group I eventually resolves into several components of which *l*, the most electronegative, separates first, whilst *m* and *n* separate only after prolonged electrophoresis, the time required increasing with the pH. Between pH 7·4 and 6·5 (Fig. 2*a*) the three constituents of group I separate easily and completely, group II shows unaltered behaviour and the subdivision of group III becomes obscure. From pH 6·50 to 5·38 (Fig. 2*b*) constituents *l* and *m* of group I separate with greater difficulty as the pH falls, whilst *k* and *j* resolve better than at higher pH values. Group III is apparently a single band.

The movement of the turbidity of the extracts can be followed by the more or less abrupt changes in blackening of the prints along the axis of migration. In general, part of the turbidity migrated with group III and at pH 8 and 7·6 entirely with *i*. Other changes in the blackening occurred at the peak of *sp* and group II, and in the cathode limb there was frequently a progressive increase in opacity from *i* to *k*.

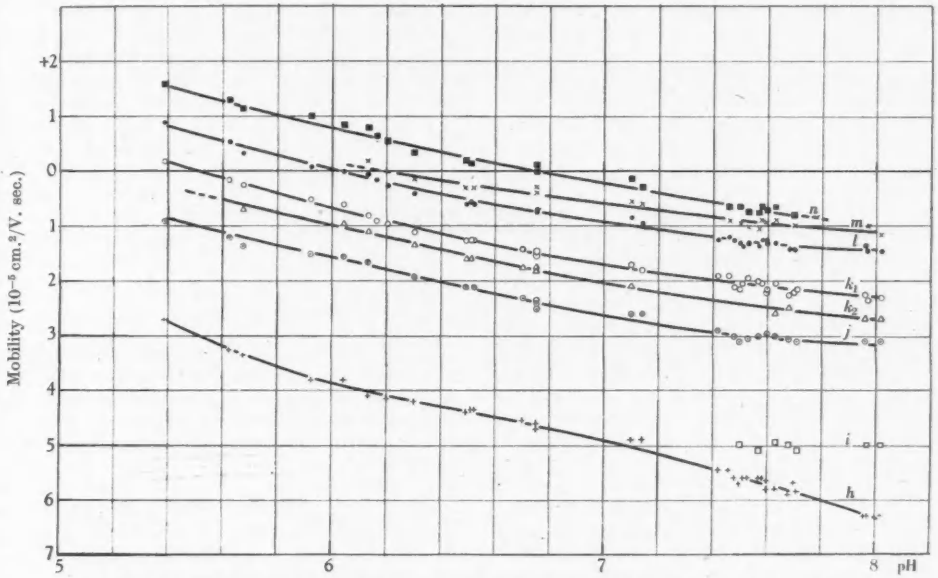

Fig. 3. Mobility-pH curves for the chief constituents of rabbit skeletal muscle, at ionic strength 0·15 ($NaH_2PO_4 + Na_2HPO_4 + 0{\cdot}05$ M-NaCl).

■ = *n* ○ = k_1 □ = *i*
× = *m* △ = k_2 + = *h*
● = *l* ⊙ = *j*

The mobilities of the principal components plotted in Fig. 3 are more accurate and complete than those of a preliminary publication (Jacob, 1945). The main objections which can be raised against the determination of the mobilities of components in a complex protein mixture will be discussed later.

The results of the measurements showing the contribution of the various constituents to the total refractive increment are given in Tables 1 and 2. Table 1 demonstrates that over a limited pH range the total nitrogen content of extracts prepared by identical methods can vary by 20 %, without

Table 1. *Concentration measurements* pH 7·10–7·8 ($\mu = 0{\cdot}15$)

Exp. no.	Rabbit no.	pH	Total N (mg./ml.)	Percentage amounts of constituents* n	m	l	k_1	k_2	j	sp	i	h
69	29	7·87	2·61†		47·5	25		11	4·5	—		—
67	29	7·85	4·29		48	22·5	8	6·5	3	2		10
23	8	7·71	5·00		50	21		13·5	5	2	4	4·5
35	12	7·70	?		45·5	27·5		11·5	3·5	—		—
21	8	7·68	4·97		48	22	8	6	4	3	3	6
1	1	7·60	4·12		48	25	9	3·5	3	2	3	6·5
38	14	7·53	3·24†		45·5	27		13·5	—	—		—
36	13	7·46	4·79		47	23		14	—	—		—
15	6	7·14	4·31	16·5	33·5	23		13	4	2		8
12	5	7·10	3·16	15	32	25		11·5	5	2·5		9
Arithmetic mean (m)‡					47·5	24		13·0	4	2·25		8·50
Standard deviation (S.D.)§					1·60	2·14		1·22	0·8	0·44		0·70
(S.D. × 100)/m					3·4	8·9		9·3	20	19		8·2

* The values are practically independent of the δ and Svensson effects (p. 90).
† Corrected nitrogen value.
‡ Values accurate to 0·25.
§ Standard deviations given by $\text{S.D.} = \sqrt{\frac{\Sigma d^2}{n-1}}$.

Table 2. *Variation of composition as a function of* pH

Exp. no.	pH	Percentage amounts of constituents n	m	l	k_1	k_2	j	sp	i	h
6	8·02	38		27	12		7·5	3·5	6	6
Experiments from Table 1*	7·10–7·87	43·5–51·5		18·5–29·5	10–16		2–6	1·15–3·35	6·75–10·25	
34	6·98	48		25	11·5		5·5	2	8	
3	6·75	15	33	25	10	3	4·5	2·5	7	
68	6·60	15	30	29	9·5	3	4·5	2	7	
17	6·53	15	29	27	14		5·5	2	7·5	
18	6·40	17	29	25·5	8·5	4·5	6	2	7·5	
37	6·30	18·5		54	8	5·5	4·5	3	6·5	
83	6·13	19		55	12		5	3	6	
82	6·04	17		55·5†	6	5·5	7	2·5	6·5	
81	5·67	18·5		53·5	15		5·5	2	5·5	

* The relative compositions given are the maximum and minimum statistical values ($m \pm 2{\cdot}5$ S.D.).
† Only experiment corrected for δ effect.

alteration of the relative quantities of the various constituents. Variations of 20% in the total concentration of extracts cannot be accounted for by such experimental variations as loss of protein through the collodion membranes, osmotic transfer of water during dialysis (Longsworth, Curtis & Pembroke, 1945), or by accidental dilution during washings. They are more probably attributable to physiological variations in the amounts of extractable protein in different rabbits. It appears that they are related to differences in the imbibing power of pulped muscles. The error of the measurements of the relative concentrations is shown by the standard deviations given in the last column but one of Table 1. The method of extrapolation used in analyzing the curves (Tiselius & Kabat, 1939) and the uncertainty in the determination of the base-line position (= 0·1 mm.) account partly for the order of the standard deviation, but the rest must be due to physiological variation.

The standard deviation for l seems very high, but for this peak the perpendicular method of extrapolation is peculiarly unsuited, since (see below) between l and m, and l and k, are components obscured at $\mu = 0{\cdot}15$. The use of the analytical method described by Svedberg & Pedersen (1940) would not in this case have resulted in much improvement. However, various measurements on the diagram from one experiment have shown that this factor does not account for the great variability of l. It would appear that for this protein the physiological variations, or the losses through the membrane, are the greatest.

In Table 2 the relative concentrations of the components are shown as a function of pH. Owing to the variation in total nitrogen of the extracts it is

not possible to compare the absolute concentrations of the components. An approximate comparison is possible for extracts from the same rabbit dialyzed at various pH values. Direct measurements have indicated that the total refractive increment and total protein nitrogen decreases on either side of the range pH 7·4–7·7. That a maximum concentration is found in this region is in agreement with the minimal precipitation observed during dialysis. The relative decrease of $m+n$ at pH 8 is due to a decrease in solubility of one of these two proteins or of a complex formed by them. Similarly the relative reduction of group III at the more acid pH values indicates a loss of solubility, probably of a progressive nature, in this group. It is not possible to establish beyond doubt whether i alone finally disappears completely or whether a complex of i and h is partially precipitated, but the former is considered more probable, as will be shown in a further publication concerned with experiments at lower pH values.

Finally, the fairly constant relative values found for other proteins as the pH decreases suggests a lowering of their absolute concentrations. It is impossible to distinguish between loss of solubility and co-precipitation as a cause of this phenomenon.

II. Experiments with varying ionic strength

The classical muscle proteins myogen, globulin X, myosin and myoalbumin may be characterized by their differing solubilities at various ionic strengths. It seemed necessary to discover to what extent the electrophoretic diagrams are modified by variation of the ionic strength of the dialysis fluid or the extraction fluid or both together. However, such experiments reveal new problems rather than clarifying the original ones, on account of the complications of interaction between proteins such as co-precipitation and mutual effects on solubility. In this investigation no more than general information of a summary character is presented.

The mobilities have been found to vary with ionic strength, but the data obtained are insufficient to warrant publication here. The measurements of relative concentration are given in Table 3.

Decrease of ionic strength

Dialysis fluid. In order to prevent the appearance of disturbing anomalies, the lowest ionic strength used was $\mu = 0{\cdot}10$, and this provides better conditions for electrophoretic separation. Thus, it is possible to resolve group I effectually (Fig. 4). After a 5 hr. electrophoresis at 7 V./cm., a peak s appears between k_1 and l in the anode limb, and after 7 hr. a further peak t separates between l and m. The total concentration of these extracts is about 80% of the corresponding extract at $\mu = 0{\cdot}15$. At corresponding pH values the relative concentrations of the constituents remain unchanged, so that a slight proportional precipitation of all proteins occurs.

Extraction fluid. If the muscle pulp is extracted with distilled water, the ionic strength of the undialyzed extract is approximately $\mu = 0{\cdot}10$, since for muscle juice $\mu = 0{\cdot}21$–$0{\cdot}26$ (Dubuisson, 1942). Rather than use large volumes of distilled water for the extraction, the ionic strength has been reduced by dialyzing the normal extract, containing about 4·5 mg. protein-N/ml. against a large volume of distilled water frequently renewed. After 4 hr. dialysis a copious precipitate was removed, a second of appreciable size after 16 hr., but after 24 hr. nothing further separated on centrifuging for 15 min. at 6000 r.p.m. The yellowish, slightly opalescent solution was dialyzed for 48 hr. against phosphate buffer pH 7·48, $\mu = 0{\cdot}15$, and contained only 2·1 mg. protein-N/ml. The electrophoresis diagrams resemble those of the ordinary extracts except that i is absent and the presence of k_2 dubious (Table 3, Exp. 51). Therefore except for i and perhaps k_2 all the constituents of the normal extracts are at least partially mutually soluble in water. The disappearance of i and k_2 would account for the loss of a maximum of 0·45 mg. protein-N/ml., but since the total loss is 2·4 mg. N/ml. the other proteins must also have been partially precipitated but in varying proportions, h the least and k_1 perhaps not at all. These facts agree with the observations of Raffel, Pait & Terry (1940) and Svensson (1941) on the electrophoretic diagrams of the euglobulins and pseudoglobulins of blood serum.

Table 3. *Concentration measurements at different ionic strengths*

Exp. no.	Ionic strength of extraction liquid	Ionic strength of dialysis liquid	pH	Percentage amounts of constituents*					
				$n+m$	l	k_1+k_2	j	sp	$i+h$
Series									
Table 1	0·15	0·15	7·10–7·87	43·5–51·5	18·5–29·5	10–16	2–6	1·15–3·35	6·75–10·25
28	0·15	0·10	7·18	51·5	19·5	13	6	2·5	7·5
32	0·15	0·10	6·83	45	26·5	12·5	5	3	8
51	0·15	0·00	7·48	43·5	28	14? 0?	3·5	3	0 8
48	0·15	0·27	7·41	43	26	12	3·5	4	11·5
60	±0·5	0·15	6·70	44·5	27·5	13·5	5	2	7·5

See note *, Table 1.

Increase of ionic strength

Dialysis fluid. It has been noted above that when an ordinary extract is dialyzed against phosphate buffer pH 7–7·6, $\mu = 0{\cdot}15$, a precipitate forms which is soluble in 0·5 M-KCl, the solution showing double refraction of flow. This material remains in solution when the extract is dialyzed against phosphate buffer pH 7·41, $\mu = 0{\cdot}27$. The dialyzed solution is much more opalescent than normal, but no material deposits on centrifuging for 15 min. at 6000 r.p.m. Electrophoresis, which is slow on account of the high ionic strength, resolves group III into three components (Fig. 5) which represent 11·5 % of the total proteins (Table 3, Exp. 48). The opalescence migrates in the anode limb with the two slower components, in the cathode with the slowest of the three peaks.

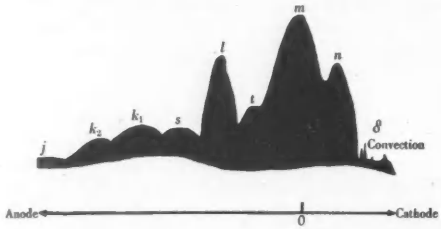

Fig. 4. Complexity of group I. Anodic pattern of a prolonged electrophoresis at ionic strength 0·10. Exp. 32: pH 6·83; electrical field, 7·20 V./cm.; duration of electrophoresis, 26,110 sec. The faster moving boundaries have left the photographic field. The base-line is distorted owing to optical imperfection of the electrophoretic cell used in this particular experiment.

Extraction fluid. Extractions at ionic strengths about $\mu = 0{\cdot}5$, which are used for isolating the myosins of Edsall (Edsall, 1939, Table 3, Exp. 60) and of Banga & Szent-Györgyi (1942), after dialysis at pH 6·75 and $\mu = 0{\cdot}15$ give diagrams similar to those of normal extracts, both qualitatively and quantitatively. The analysis of the curves is difficult because of the low protein content, 1·542 mg. N/ml. for Exp. 60, and 0·882 mg. for Banga-Szent-Györgyi extracts. The physico-chemical and biological problems which these experiments present should be examined under more favourable conditions.

Extraction and dialysis fluids. Extraction fluid: 0·048 M-Na_2HPO_4, 0·006 M-NaH_2PO_4, 0·2 M-NaCl, $\mu = 0{\cdot}35$, pH = 7·45. Dialysis fluid: 0·032 M-Na_2HPO_4, 0·004 M-NaH_2PO_4, 0·25 M-NaCl, $\mu = 0{\cdot}35$, pH = 7·45.

These dialyzed extracts are very turbid and the electrophoresis diagram markedly altered (Fig. 6), but groups I, II and III are distinguishable. The component *sp* is present in much larger proportion than at lower ionic strengths, for a comparable migration and height of group III, and completely masks *i*. Group II is atypical in appearance and is characterized by a very sharp peak in the position usually occupied by *j*. In the anode limb the turbidity coincides precisely with this peak, whilst in the cathode limb it is situated between *i* and *k*. Other less well-defined asymmetries occur and relative concentration measurements were impracticable.

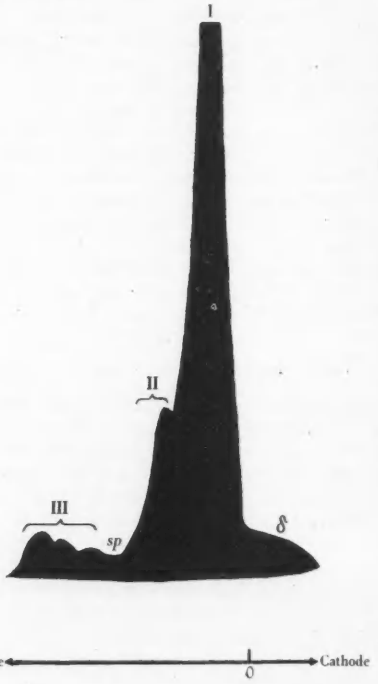

Fig. 5. Emergence of a new constituent in group III in muscle extracted at an ionic strength of 0·15 but dialyzed at an ionic strength of 0·27. Exp. 48: anodic pattern; pH 7·41; electrical field, 4·12 V./cm.; duration of electrophoresis, 13,860 sec.

Increasing the ionic strengths of the extraction and dialysis fluids further involves electrophoresis runs of 24 hr. duration at least, which involves increased risks with respect to convection and diffusion effects. For the present a limit of ionic strength $\mu = 0{\cdot}35$ has been imposed.

Experiments at various pH values with acetate buffers as extractants have been made; owing to the buffering capacity of the pulp, these have up to the present merely been confirmatory of the results obtained on the effect of the pH of the dialysis fluid.

III. Technique of analysis of the diagrams

In order to define the physico-chemical limitations of the moving-boundary method, the effect of errors resulting from thermal convection, the configurational asymmetries between ascending and descending boundaries and the effect of various factors on mobility, have been experimentally examined. The main theoretical and experimental developments will be found in the papers of Tiselius (1930, 1937), Kekwick (1940), Longsworth (1941, 1942), MacInnes & Longsworth (1944), and Overbeek (1943), and only a brief discussion is necessary here.

peak preceding h, *sp* and δ, or ϵ. In the first of these, convections occur only in the cathode limb, irregularly at $\mu = 0{\cdot}15$ and more frequently at higher ionic strengths (Fig. 6). After 2–3 hr. electrophoresis, *sp* is affected only on the cathode side; after 6–7 hr. δ becomes deformed (Figs. 1*b*, 4), and ϵ is never changed. All these convections disappear if the current is cut off for 1–1½ hr. It follows that the appearance of convection disturbances is not solely due to small density gradients, but a contributory condition is that the gradient should occur in a region of relatively high protein concentration, the viscosity of which opposes the dispersion of the convection by normal diffusion. Practically, these

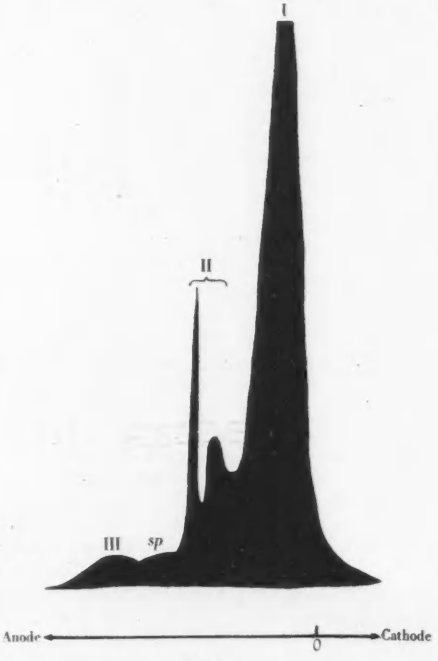

Fig. 6*a*.

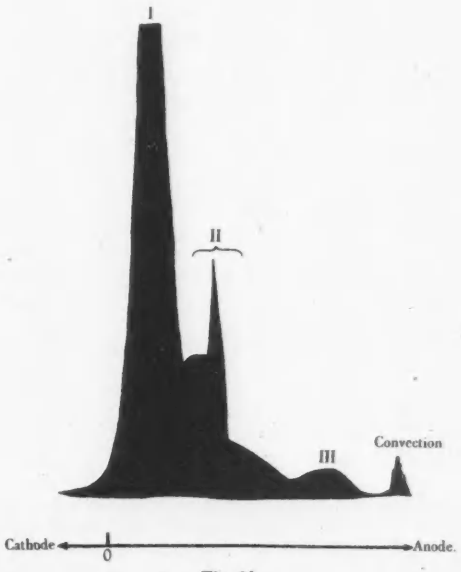

Fig. 6*b*.

Fig. 6. Appearance of *sp* and group II in muscular extracts obtained and dialyzed at an ionic strength of 0·35; (*a*) anodic; (*b*) cathodic. Exp. 74: pH 7·45; electrical field, 1·75 V./cm.; duration of electrophoresis, 29,000 sec. Convection means convection disturbances (see p. 89).

Thermal convection

When the vertical gradient of density, the principal stabilizing factor in the moving boundary method, is very small, convection is unavoidable (Shedlovsky & Smadel, 1940; Longsworth, 1942). Considering the present extracts, there are three regions in which low-density gradients may develop after varying periods of electrophoresis; the broad disturbances are not of serious importance, and do not influence the mobility of adjacent components.

Asymmetry between ascending and descending boundaries

The main asymmetries are (Longsworth & MacInnes, 1940): (*a*) the δ and ϵ effects, (*b*) the sharper form and faster migration of the ascending boundary.

(*a*) The δ and ϵ effects will only be considered at $\mu = 0{\cdot}15$ (Table 4). Above pH 7·1 these boundaries separate satisfactorily from the protein boundaries, and agreement between the percentages of the components in both limbs is obtained by excluding δ and ϵ (Table 4). The effect of ϵ is negligible and will not be further considered.

peaks, an almost isoelectric iso-δ peak and descending peaks. The ascending peaks, cathode I and anode *j*, *sp*, III, are decreased by the partial δ peak, and the iso-δ peak is increased by the sum of these effects which is greater in the cathode limb, since I accounts for 72 % of the total protein, than in the anode limb, where *j*, *sp*, and III together account for

Table 4. *Comparison of constituents in anode and cathode limbs*

Exp. no.	pH	mg. N/ml.	Group I		*k*		*n*+*m*				*l*	
			Anode	Cathode	Anode	Cathode	Anode		Cathode		Anode	Cathode
69	7·87	2·612	72·5	72·5	11	11	47·5		44		25	28·5
67	7·85	4·294	70·5	73	14·5	12	48		48		22·5	25
21	7·68	4·974	70	72·5	14	15	48		50		22	22·5
1	7·60	4·120	73	72	12·5	14	48		—		25	—
							n		*m*			
							Anode	Cathode	Anode	Cathode		
15	7·14	4·318	73	73	13	12	16·5	—	33·5	—	23	30
12	7·10	3·160	72	73·5	11·5	11	15	13	32	30	25	30·5
3	6·75	3·320	73	72·5	13	11·5	15	19·5	33	21·5	25	31·5
60	6·70	1·542	72	74	13·5	11	14·5	15·5	30	17·5	27·5	41
68	6·60	3·472	74	73	12·5	11·5	15	15	30	29	29	29
									m+*l*			
									Anode		Cathode	
37	6·30	3·092	72·5	73·5	13·5	14·5	18·5	18·5	54		55	
83	6·13	?	74	73	12	12·5	19	18	55		54·5	
82	6·04	?	74·5	66·5	11·5	16·5	17	15·5	57·5		51	
81	5·67	?	72	63	15	24	18·5	16·5	53·5		46·5	

In the pH range studied, the anodic and cathodic percentages of components *j*, *sp* and III are concordant within experimental error.

Below pH 7·14, the δ effect merges with the protein diagram, and to simplify the problem, consider first the diagrams in which group I is still represented by a single peak (Table 4, left-hand side). Close to pH 7·10, the δ boundary merges partially with the anode band of group I, so that a portion of it can be excluded. From pH 6·75 to 6·13 it is completely masked by group I (anodic), but the anode and cathode percentages of the constituents of this group are in agreement. Group I is therefore increased on the anode side by the effect, the magnitude of which is less than possible experimental error. As a first approximation this δ effect is caused by group II, *sp* and group III, that is, 28 % of the total protein of the extract, and is accordingly of little significance theoretically (Longsworth, 1942), and neither is the error introduced in the estimates of II, *sp* and III.

From pH 6·04 to 5·67 an important δ effect interferes increasingly with the band *k*, migrating to the cathode, and the disagreement in the analyses is considerable.

The values obtained at pH 5·67 can be accounted for quantitatively if the anode and cathode diagrams are each considered as mixed, containing ascending only 13 % of the total protein. The descending peaks are subject only to a negligible correction. At pH 6·04 the same argument can be applied taking *l* and *k* to be iso-δ boundaries.

Considering the asymmetries which exist within group I, no difficulties arise above pH 7·14 or below pH 6·13. Within the range pH 6·13–7·14 the situation is complex, and it seems necessary to consider the possible occurrence of protein interaction similar to that found for egg-white (Longsworth, Cannan & MacInnes, 1940), and for ovalbumin-yeast nucleic acid mixtures (Longsworth & MacInnes, 1942). It must be pointed out that at certain pH values the iso-δ boundaries can be different in the anode and cathode limbs because the conditions governing the migration of the δ boundary are not identical in the two limbs.

The Svensson effect (Svensson, 1943) revealed in the asymmetry of leading peaks has no measurable effect on the data obtained at $\mu = 0{\cdot}15$, since the percentage composition obtained at this ionic strength has been independent of the total protein concentration (Table 4, 69–60). Further, the composition does not change if the ionic strength is varied, provided that those proteins insoluble at

$\mu = 0{\cdot}15$ have been precipitated previously during preliminary dialysis. There is no appreciable Svensson effect because the rapidly migrating components are present at low concentrations, and consequently modify very slightly the solution through which they pass and are not themselves affected by other gradients.

(*b*) The ascending peaks are generally better separated and sharper than the descending except for *h* which is more sharp in the cathode than anode; but this may be a viscosity effect. In Table 5 are given the mobilities, both ascending and descending, of those constituents *h*, k_1 and *l* for which accurate measurements are possible. The interpretation of these data must be considered in relation to the mobilities and the errors in their measurement. The mobilities have been evaluated by the accepted methods and are apparent values, since the potential gradient has been taken as that in the initial protein solution and not the actual gradient occurring at each boundary. The errors occurring in these determinations do not have the same significance in comparing the ascending and descending mobilities of different bands during the course of a single experiment, or in comparing the results of different experiments.

In the first case the relevant errors which occur are: (1) in the calibration of each compartment of the cell; (2) in the determination of the position of the peak (this error can vary according to the peak considered, and is a function of duration and voltage) and (3) in the compression of the vaseline at the lower plate joint which causes a negligible increase of 'classical' asymmetry (see Table 6).

Table 5. *Comparison of apparent mobilities of ascending and descending boundaries for three constituents*

			$u \times 10^5$									
			h			k_1			*l*			
Exp. no.	pH	mg. N/ml.	Asc.	Desc.		Asc.	Desc.		Asc.	Desc.		
6	8·02	3·90	−6·38	−6·26	A*	−2·31	−2·32	—	−1·48	−1·44	—	
4	7·96	2·99	−6·30	−6·32	—	−2·23	−2·23	—	−1·45	−1·35	A	
35	7·70	?	−5·72	−5·72	—	−2·30	−2·18	A	−1·53	−1·39	A	
21	7·68	4·97	−5·92	−5·94	—	−2·29	−2·23	—	−1·48	−1·41	—	
22	7·63	5·01	−5·86	−5·80	—	−2·16	−2·03	A	−1·43	−1·28	A	
1	7·60	4·12	−5·70	−5·65	—	−2·18	−2·18	—	−1·35	−1·25	A	
41	7·60	2·00	−5·87	−5·80	—	−2·25	−2·14	A	−1·41	−1·31	A	
2	7·58	3·15	−5·58	−5·60	—	−2·09	−2·04	—	−1·26	−1·23	—	
24	7·57	5·17	−5·80	−5·60	A	−2·24	−1·98	A	−1·48	−1·33	A	
38	7·53	3·25	−5·57	−5·62	—	−2·13	−1·95	A	−1·43	−1·30	A	
42	7·51	1·21	−5·83	−5·63	A	−2·05	−2·03	—	−1·34	−1·34	—	
40	7·50	2·83	−5·74	−5·70	—	−2·25	−2·13	A	−1·43	−1·32	A	
51	7·48	?	−5·64	−5·67	—	−2·10	−2·09	—	−1·35	−1·25	A	
36	7·46	4·79	−5·43	−5·47	—	−2·04	−1·87	A	−1·37	−1·18	A	
50	7·42	?	−5·75	−5·45	A	−2·12	−1·92	A	−1·43	−1·23	A	
15	7·14	4·30	−4·90	−4·89	—	−1·78	−1·80	—	−0·95	−0·95	—	
12	7·10	3·16	−4·87	−4·89	—	−1·78	−1·68	A	−0·93	−0·79	A	
3	6·75	3·32	−4·50	−4·58	—	−1·49	−1·51	—	−0·75	−0·73	—	
10	6·75	2·42	−4·93	−4·73	A	−1·64	−1·50	A	−0·87	−0·75	A	
11	6·75	2·12	−4·65	−4·60	—	−1·47	−1·43	—	−0·68	−0·68	—	
60	6·70	1·54	−4·62	−4·56	—	−1·41	−1·40	—	−0·77	−0·65	A	
17	6·52	4·60	−4·38	−4·36	—	−1·36	−1·26	A	−0·70	−0·60	A	
18	6·49	4·34	−4·44	−4·42	—	−1·34	−1·27	—	−0·70	−0·60	A	
37†	6·30	3·09	−4·18	−4·20	—	−1·11	−1·10	—	−0·53	−0·51	—	
83†	6·13	?	−4·08	−4·12	—	−0·85	−0·81	—	−0·05	−0·06	—	
80†	5·92	?	−3·83	−3·83	—	−0·51	−0·47	—	+0·08	+0·08	—	
81‡	5·67	?	−3·55	−3·35	A	−0·25	−0·25	—	+0·50	+0·35	A	
79‡	5·62	?	−3·33	−3·26	—	−0·16	−0·12	—	+0·70	+0·55	A	
78‡	5·38	?	−2·71	−2·69	—	+0·25	+0·22	—	+1·13	+0·90	A	

* A indicates that the asymmetry exceeds the experimental error.

† Experiments near the apparent I.E.P. of *l* (pH 6·00±0·05). There is no asymmetry for *l* in this case. Thus, these experiments are not concerned in the calculation of the asymmetries of boundary *l* (Table 5).

‡ I.E.P. of k_1 pH 5·50±0·05. No asymmetry.

In comparing the results from different experiments, errors in conductivity determinations, pH and current measurement must also be considered. There are other systematic errors due to liquid displacement (Tiselius, 1930), and accidental errors which Longsworth (1943) shows to be possible with even the best experimental arrangement. In this latter case an agreement of $\pm 0{\cdot}10 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$ is excellent and is not exceeded by most of our results (Fig. 3). Thus, in the former case, we have to consider an error of approximately $\pm 0{\cdot}05 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$, and owing to the relatively great

number of experiments it is likely that the asymmetry increases from h to k and from k to l (Tables 5 and 6). In every case it has been verified that this gradation cannot be due to accidental occurrences such as convexion currents at sp on the cathode side, or to interaction between l, m and n. It seems to be correlated with the increase in the relative concentrations of the components h (5–6 %), k_1 (8–10 %), and l (22–28 %). It follows that in the electrophoretic separation of a mixture of proteins, the velocity asymmetry is greatest for the component of highest relative concentration.

Table 6. *The asymmetries of the boundaries h, k_1 and l*

Boundary	h	k_1	l
Frequency of asymmetry*	0·21	0·42	0·73
Mean deviation†	+0·045	+0·071	+0·10
Mean deviation as % of mobility‡	+1 %	+4 %	+8 %

* Frequency of asymmetry is the ratio of the number of cases of asymmetry (A, Table 5) to the total number of experiments.

† The difference between ascending mobility and descending mobility is taken as positive when ascending > descending (classical asymmetry).

‡ Mean deviation as percentage of mobility of descending boundary, near pH 7·6.

The factor of individual concentration seems to be important. For h there is practically no asymmetry, and even if the errors in all experiments were such as to increase the classical asymmetry to a maximum ($2 \times 0{\cdot}05 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$) the mean deviation as percentage of mobility should be +3 %, and in only five out of twenty-nine experiments has this value been exceeded. The component h therefore migrates nearly ideally without change in its concentration throughout the liquid column, which is remarkable, since above pH 6·75 it migrates in a more varied medium than any of the other components.

In practice the medium modifies during the course of electrophoresis (Longsworth & MacInnes, 1940), and during the first hour the mobilities of the ascending and descending boundaries of h may differ by *c.* 10 % but become equal after 2–3 hr. This may be a consequence of the regulation function of Kohlrausch (1897), studied in relation to salt ions by MacInnes & Longsworth (1932).

It may be concluded, that in the electrophoresis of a protein mixture at favourable ionic strength ($\mu = 0{\cdot}15$) self-regulation occurs most markedly for the constituents of lowest concentration. This regulation which tends to equalize the apparent mobilities in the ascending and descending limbs introduces qualifications to the conclusions of Longsworth & MacInnes (1940). These conclusions are, however, a satisfactory approximation, and since we have not observed a negative asymmetry in conformity with the predictions of these authors, their view has been adopted and the values have all been taken from the descending limb in constructing the curves of Fig. 3.

Miscellaneous factors

The mobility measurements have proved to be substantially independent of a number of factors which theoretically might possibly have had an effect. This is particularly true of the total protein concentration (Table 7) which would be expected to

Table 7. *Independence of mobility with respect to total concentration of extract*

Exp. no.	mg. N/ml.	pH	Descending mobility h	k_1	l
42	1·214	7·51	5·65	2·05	1·35
40	2·832	7·50	5·70	2·15	1·30
22	5·016	7·63	5·80	2·05	1·30

influence the viscosity of the medium through which the boundaries migrate, particularly h in the cathode limb, and so alter the mobility. These observations disagree with those of Longsworth & MacInnes (1940) on ovalbumin, but agree with the findings of Stenhagen & Teorell (1939) on thymonucleic acid. The author agrees with the latter's distinction between real viscosity which enters into the electrophoretic equation, and apparent viscosity called plasticity which depends on protein concentration and does not affect the mobility.

The magnitude of the potential gradient has no measurable influence (Table 8) which is contrary to

Table 8. *Independence of mobility with respect to potential gradient and length of dialysis*

Exp. no.	V./cm.	Length of dialysis (hr.)	mg. N/ml.	pH	Descending mobility h	k_1	l
23	2·86	88	5·002	7·70	5·85	2·15	—
21	4·22	40	4·974	7·68	5·95	2·25	1·40
22	6·76	64	5·016	7·63	5·80	2·05	1·30

the findings of Stenhagen & Teorell (1939) who obtained a 3 % increase in mobility for unit increase in potential gradient. Exp. 22 should have furnished a measurable effect

$$\left(5{\cdot}85 + \frac{3 \times 4}{100} \times 5{\cdot}85 = 6{\cdot}55\right)$$

according to the above results.

The temperature increase in a potential gradient of 5 V./cm., determined with thermocouples is

0·2–0·3°, which would have a negligible effect on the viscosity and the conductivity of the solution. Duration of dialysis has no effect provided it is sufficient but not so prolonged as to cause denaturation of certain of the proteins. Thus muscle proteins do not behave like haemocyanins (Tiselius & Horsfall, 1939). Reversible electrodes of the $Cu/CuSO_4$ or Ag/AgCl type may be used indiscriminately.

In conclusion, the mobility curves given characterize proteins in mixtures where one component may influence another, and are likely to be useful in the identification of constituents ultimately obtained pure.

DISCUSSION

In extracts of striated rabbit muscle freed from blood, there exist above pH 7·10 and at ionic strengths 0·10–0·15 at least eleven electrophoretically distinguishable components. It is difficult to establish the exact relationship between these components and the classical muscle proteins, but there are some points worth indicating. At an ionic strength of 0·15, the isoelectric points (I.E.P.) of the group I proteins are *n* 6·75, *m* 6·20, *l* 6·00, and for *t* and *s* close to pH 6·00. The soluble portion of group I seems to correspond with the myogen of Weber (1934) (I.E.P. 6·3) which has been known for some time to be a mixture of proteins (Finn, 1932; Baranowski, 1941). This identification is supported by the findings of Bate Smith (1940). It is not possible to place zymohexase precisely.

Component *h*, perhaps totally water-soluble, may be myoalbumin, and represents 7–8 % of the water-soluble proteins. At pH 5·38 it still has a high anodic mobility in agreement with Bate Smith (1937). For myoalbumin at pH 7 and $\mu = 0{\cdot}1$ Bate Smith (1940) gives a mobility which can correspond only with *h*. The other components (k_1, k_2, *j*, *sp* and *i*) may represent myosin and globulin X, whose isoelectric points are near pH 5·00 (Weber, 1934). It may be objected that at ionic strength $\mu = 0{\cdot}15$, the myosins of Edsall and of Banga & Szent-Györgyi are not sufficiently soluble to appear in the present diagrams. However, Bate Smith (1937) has demonstrated that it is difficult to precipitate the myosin of Edsall from solutions containing phosphate, and further it is probable that in addition to the effect of ions the presence of other proteins affects the solubility. Of k_1 little is known except that its isoelectric point is 5·50. The components k_2, *j*, *sp* and *i* are accompanied at $\mu = 0{\cdot}15$ by a turbidity the distribution of which is difficult to explain. Of these four proteins, *i* is at present the best characterized by its insolubility in distilled water, and complete loss of solubility below pH 6·0. They are important since it is in this region of the diagram that changes occur when the protein responsible for birefringence is kept in solution, or those proteins more soluble at $\mu = 0{\cdot}35$ than at $\mu = 0{\cdot}15$ are investigated. These last considerations suggest that electrophoretically there is not one but several myosins, if myosin is defined as a globulin much more soluble at $\mu = 0{\cdot}27$–$0{\cdot}35$ than at $\mu = 0{\cdot}15$. This disagrees with the results of Bailey (1942) but agrees with those of Ziff & Moore (1944).

It is possible that the electrophoretic method may introduce important corrections to the existing classification of muscle proteins, but further experiments are necessary to establish this. In a previous publication (Jacob, 1945), the main differences between the muscle proteins of the rabbit and frog have been described. In a private communication, J. W. Mehl has indicated that extracts of rat muscle are similar electrophoretically to those of rabbit.

SUMMARY

1. Saline extracts of striated rabbit muscle have been examined in the Tiselius-Longsworth electrophoresis apparatus.

2. In the extract obtained with phosphate of pH 7·70 (0·048 M-Na_2HPO_4–0·06 M-NaH_2PO_4, $\mu = 0{\cdot}15$) and dialyzed against phosphate buffer containing 0·05 M-NaCl, total $\mu = 0{\cdot}15$, there are nine components divisible into the following three groups: group I consisting of three proteins, *n*, *m* and *l*; group II, three proteins, k_1, k_2 and *j*; group III, which at pH 7·4 and above contains two proteins *h* and *i*, and below pH 7·4 is apparently homogeneous; between groups II and III is found a heterogeneous component *sp*.

3. The mobility curves of the first eight components have been established at ionic strength $\mu = 0{\cdot}15$ over the pH range 5·38–8·04. From these the following isoelectric points have been interpolated: *n*, $6{\cdot}75 \pm 0{\cdot}05$; *m*, $6{\cdot}20 \pm 0{\cdot}05$; *l*, $6{\cdot}00 \pm 0{\cdot}05$; and k_1, $5{\cdot}50 \pm 0{\cdot}05$.

4. Some of the constituents show marked variations in solubility over the pH range studied.

5. At an ionic strength $\mu = 0{\cdot}1$, two further proteins resolve in group I.

6. When the extracts are dialyzed against distilled water the protein *i* is apparently completely precipitated.

7. Extraction or dialysis at higher ionic strengths ($\mu = 0{\cdot}27$–$0{\cdot}35$) shows that the proteins with higher solubilities in this range migrate close to k_1, *j*, *sp* and *i*.

8. Various aspects of technique apposite to the study of the protein mixtures have been examined experimentally. These include: the effects of thermal convection; the δ boundary and Svensson effect and their significance in the measurement of relative concentration; asymmetries in the ascending and descending mobilities of three of the consti-

tuents of the mixtures; and the influence of extract concentration, potential gradient, and duration of dialysis on the mobility.

9. The relation between the observed electrophoretic components and the classical muscle proteins has been examined.

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Studies in Vitamin A

1. A CHROMATOGRAPHIC METHOD FOR SEPARATING FREE AND ESTERIFIED VITAMIN A

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Partition between immiscible solvents, usually light petroleum and aqueous methanol, has long been in use to separate carotenoid hydrocarbons from hydroxyl-containing 'xanthophylls'. Gillam & Senior (1936) found that in the absence of much cholesterol, vitamin A alcohol could be removed from light petroleum by seven extractions with 90% aqueous methanol. Reed, Wise & Frundt (1945) effected a partial separation of free and esterified vitamin A by extracting light petroleum solutions with 95% aqueous methanol; 98% of the esterified and 27% of the free vitamin remained epiphasic and simple equations permitted the proportions of combined and free vitamin to be calculated. As in the earlier work of Gillam & Senior (1936), the presence of cholesterol adversely affected the separation. This interference is a serious drawback to the use of partition methods in that biological

materials, e.g. blood lipids, contain cholesterol in much greater amount than vitamin A. Claims have also been made that free vitamin A is removed from vitamin A-bearing oils by washing with methanol (e.g. Reti, 1935; Ritsert, 1942).

A fluorimetric method (Sobotka, Kann & Winternitz, 1944) makes use of differences in rates of development and fading of fluorescence to distinguish between free and esterified vitamin A. Quantitative estimations are possible with simple mixtures of purified ester and vitamin A alcohol, but unsaturated glycerides which normally accompany the vitamin, interfere to a serious extent. Chromatographic separations on alumina have been reported by Müller (1944) and Reed *et al.* (1945). Müller used long columns consisting of successive layers of alumina preparations graded in respect of adsorptive activity, but found them difficult to reproduce. Reed *et al.* used short columns of alumina and developed and eluted the chromatogram with dichloroethylene; the method appears to be effective but difficulty in procuring the solvent has limited its use. The adsorbent, however, may affect the vitamin so as to form from it an orange red pigment (Castle, Gillam, Heilbron & Thompson, 1934) which is considered by Meunier & Vinet (1944) to be the ether derived from two molecules of free vitamin A ('biaxerophthyl ether'). We have ourselves often noticed the formation of such an artefact during protracted chromatography on alumina. The change may occur on other adsorbents (Holmes & Corbett, 1939) but of all those we have tried, bone meal is the least open to suspicion. Only a short column of extracted bone meal is needed for rapid and clean separations of some carotenoid mixtures (Mann, 1943; Goodwin & Morton, 1946) and only small volumes of solvents are needed. It therefore seemed likely that vitamin A alcohol could be conveniently separated from its esters by chromatography on bone meal. The method employed is described in the present paper.

EXPERIMENTAL

Preparation of adsorbent and column. Defatted bone meal (10 g.) obtained by extracting commercial bone meal with acetone for 6 hr. in a Soxhlet and drying at 60°, is sieved on no. 120 B.S. and no. 200 B.S. sieves. The fraction passing the 120 and retained by the 200 sieve is used for the chromatogram, and 5 g. are transferred to a chromatograph tube 15 cm. × 15 mm. diameter constricted at its base to 5 mm. diameter. The constriction is firmly plugged with cotton wool, and the column connected to a Buchner flask so that suction can be applied to aid elution.

Operation of the column. The test material is made up in light petroleum (b.p. 40–60°) to a concentration of 5–15 mg./100 ml. The bone meal is first wetted and well covered with light petroleum, then stirred with a glass rod to remove any trapped air. It is then tamped down firmly while gentle suction is applied. From this point onwards the column must *on no account* be allowed to run dry. The vitamin solution (5 ml.) is now added and the ester fraction eluted by washing through with approximately 100 ml. of 2·5% (v/v) chloroform in light petroleum. The receiver is changed and the adsorbed vitamin A alcohol is eluted with 50–60 ml. pure redistilled acetone. Occasionally this eluate is turbid, owing to some 'fines' from the column brought through with acetone. These particles are easily removed by filtration through a G4 sintered glass crucible.

The solvents are removed from the two fractions on a warm water-bath, the last traces of solvent being removed in a stream of nitrogen. The residues are redissolved in chloroform and examined for vitamin A by the Carr-Price colour test.

The free vitamin A when adsorbed on bone meal is extremely unstable and in order to reduce oxidative losses during chromatography it is essential to elute this fraction without delay and without allowing the adsorbent to become dry at any stage in the separation. Under these conditions only very small losses of free vitamin A occur; the esterified vitamin A is not so susceptible to destruction on the column.

At the end of an experiment any residual acetone on the column is removed by washing with 50 ml. light petroleum and the column is ready for use again. Columns treated thus can be used many times, but it is important to re-test them periodically with pure solutions of vitamin A alcohol and ester. The adsorptive powers of columns which have been used with unsaponifiable fractions of oils deteriorate more rapidly than of those used to fractionate whole oils.

Determination of vitamin A

All reported values were obtained by measuring the intensity of absorption at 617 mμ of the blue colour produced when vitamin A reacts with antimony trichloride in chloroform solution. Intensities were measured by means of a Beckman spectrophotometer (Cary & Beckman, 1941). As the colour produced in the $SbCl_3$ reaction is highly transitory the intensities were measured between 5 and 10 sec. after mixing the reactants. A sample of the test solution (0·3–0·5 ml.) is placed in the absorption cell, which is already in position in the cell holder, and two drops of acetic anhydride added as a precaution against turbidity. The antimony trichloride reagent (3 ml.) is then run into the cell from a quick-flowing burette. The cell holder is quickly placed in position in the instrument (already set and balanced at 617 mμ), the 'light tight' lid shut and the phototube switched into the light beam. The maximum intensity recorded is noted. With practice one person can complete all these operations within 5 to 10 sec. of mixing. The concentrations of the test solutions were adjusted to give extinctions of the order 0·3–0·8, over which range the instrument is most accurate. Under these conditions the vitamin A potency of a solution can be determined within ∓2%.

Calibration experiments. The free vitamin A used was obtained from two sources: (*a*) a concentrate

containing 30% vitamin A, being the residue from the crystallization of vitamin A, and (*b*) the unsaponifiable fraction from a rich fish liver oil. The ester concentrate used was produced by molecular distillation. Samples of these concentrates in light petroleum solutions were mixed and subjected to chromatographic separation. The results quoted in Table 1 show the separation obtained for a typical series of experiments and demonstrate that recovery of either fraction is generally within 5% of that expected.

Table 1. *Chromatographic separation of vitamin A alcohol and esters*

	Vitamin A ester			Vitamin A alcohol		
Solution	Added (i.u.)	Recovered (i.u.)	Recovery (%)	Added (i.u.)	Recovered (i.u.)	Recovery (%)
A	7030	7070	100·8	Nil	Nil	—
B	Nil	Trace	—	2100	2016	96·0
C	1206	1140	94·5	255	252	98·9
D	1482	1450	97·9	1330	1302	97·9
E	1482	1418	95·6	2660	2410	91·8
F	1020	940	92·1	1300	1240	95·3

Table 2. *Recovery and separation of free and combined vitamin A added to liver extracts*

Original liver extract			Fortified extract			
			Free vitamin A (i.u.)		Esterified vitamin A (i.u.)	
Free vitamin A (i.u.)	Esterified vitamin A (i.u.)	Vitamin A added (i.u.)	Expected	Found	Expected	Found
310	4040	3920 (ester)	310	280	7960	8160
182	1810	1540 „	182	191	3350	3270
182	1810	160 (free)	342	329	1810	1770
67	3360	168 „	235	233	3360	3360

These results were obtained using highly purified solutions and it remained to be seen if the technique was adequate to deal with biological extracts, especially rat liver lipids and blood lipids in which the amount of sterols present greatly exceeds that of vitamin A. To test this point a number of rat liver extracts was examined both before and after addition of small quantities of free and combined vitamin A. Table 2 summarizes the results obtained which show that the separation is entirely satisfactory in presence of sterols in considerable quantities.

SUMMARY

1. A chromatographic method of separating vitamin A alcohol and esters has been devised in which defatted bone meal is used as adsorbent.

2. The method is satisfactory in the presence of large amounts of sterols.

3. Recoveries of 95% of either fraction can be obtained if the experimental conditions described are rigorously followed.

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Studies in Vitamin A

2. THE RELATIONSHIP BETWEEN BLOOD VITAMIN A LEVELS AND LIVER STORES IN RATS

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Variations in the vitamin A content of blood plasma have been the subject of much study. Neither in man nor in animals has any simple relationship been demonstrated between plasma levels and the amounts of vitamin stored in the liver. It is not necessary here to discuss all the data in which blood and liver determinations on individual animals were made, but brief reference to a few findings will suffice to put the problem in its setting.

Steigmann & Popper (1944) claim that if the results for a large number of individuals are considered, a rough parallelism is observed between blood and liver levels. The data of Lewis, Bodansky, Falk & McGuire (1942), however, carry the implication that plasma levels are controlled by a mechanism independent, to some extent at least, of the magnitude of the liver 'reserve'. In rats, the plasma levels remained between 37–53 i.u./100 ml. even when the animals received too little dietary vitamin A to allow accumulation in the liver. Ralli, Bauman & Roberts (1941) and Josephs (1942) approached the problem from another direction and found that post-absorptive plasma levels in man and the rat were only slightly raised after large doses of vitamin A. The literature as a whole indicates the existence of a mechanism tending to resist change in plasma levels despite gross variations in vitamin A intake.

Gray, Hickman & Brown (1940) and Gray & Cawley (1942) fed vitamin A as free alcohol and as ester to rats. They concluded that both found their way to the liver to be stored as ester, but that analytical molecular distillation always revealed the presence in liver of very small amounts of vitamin as free alcohol. The vitamin A circulating in the blood is normally the alcohol (Clausen, Baum, McCoord, Rydeen & Breese, 1942). It therefore seems possible that *the liver level of unesterified vitamin is the variable controlling plasma levels*. Since the determination of vitamin A in small amounts of plasma has been mastered and a chromatographic separation of free and esterified vitamin A worked out (Glover, Goodwin & Morton, 1947), it became possible to test the above hypothesis.

EXPERIMENTAL

Estimation of vitamin A. Vitamin A was determined photoelectrically using the absorption maximum (617 mμ) in the antimony trichloride colour test. The instrument (Beckman spectrophotometer) gives readings of high accuracy at E values from 0·2 to 1·2, with 0·4 near the optimum as compared with 2·0 on a visual instrument. The amount of plasma needed is thus reduced to one-fifth and it was often possible to obtain enough plasma from a single rat for duplicate assays.

The rats used in most of the tests were males 6–9 months old reared on a balanced diet in the form of special rat cubes (Lever Brothers and Unilever Ltd.). This food contains a little vitamin A but not enough to give rise to significant pre-experimental variations in vitamin A intake. The rats were divided into groups of three and were dosed orally by dropping 1 ml. of oil on to the back of the tongue from a small syringe. The oils were either tested fish liver oils or concentrates diluted to a definite potency with arachis oil.

In order to obtain rats with very low reserves of vitamin A, newly weaned animals were given the following vitamin A-free diet for 7–9 days: casein 20%, rice starch 60%, lard 10%, salt mixture 5%, marmite 5%.

Collection and extraction of blood. At a stated time after dosing (3–14 days) and not less than 12 hr. after the last feeding, the rats are anaesthetized with chloroform and a small flap of skin reflected over the sternum. Blood is drawn after cardiac puncture into a syringe moistened inside with heparin solution. The sample is transferred to a centrifuge tube and the plasma separated by spinning. The plasma is poured into a measuring cylinder and then transferred to a small separating funnel. An equal vol. of ethanol is introduced into the same cylinder, and after stirring is added slowly and with constant shaking to the plasma (cf. Yudkin, 1941). Vitamin A is then extracted by shaking twice with an equal vol. of light petroleum (b.p. 40–60°). The combined extracts are freed from solvent on a warm water-bath and the residue made up to an appropriate vol. with ethanol-free chloroform.

Extraction of liver tissue. The freshly removed liver is freed from adhering tissue and weighed. It is then finely ground in a mortar with approx. 5 times its weight of anhydrous Na_2SO_4 and a little acid-washed silver sand. The homogenized mixture is transferred to a 250 ml. conical beaker and extracted once with 150 ml. and then twice with 75 ml. portions of ethyl ether freshly redistilled

over reduced iron. Extraction is facilitated by rapid stirring of the contents of the beaker on a warm water-bath for 2 min. The beaker is allowed to stand for a few minutes when most of the ether may be decanted, and the remainder removed by filtration through a Buchner funnel. The three extracts are combined and the ether removed on a water-bath, the last traces in a stream of nitrogen. The residue is dissolved in light petroleum (b.p. 40–60°) and examined for free and combined vitamin A as previously described.

RESULTS

The data sought for single animals, or sometimes groups of similarly dosed animals, were (*a*) plasma vitamin A, (*b*) free vitamin A in liver, and (*c*) esterified liver vitamin A. The liver storage for animals receiving very large doses fell below expectations but the effect has not yet been fully studied. The main findings are collected in Table 1. The highest dosage was in the form of an arachis oil solution of a rich (30%) vitamin alcohol concentrate (British Drug Houses Ltd., molecular distillate), but apart from this ester preparations were used. The figures confirm the observation of Gray *et al.* (1940) that vitamin A, whether fed as free alcohol or ester, is stored in the liver overwhelmingly as ester.

Livers of rats with small vitamin A stores contain only minute amounts of the alcohol. In such cases the slight losses which occur in the alcohol fractions during the most careful chromatography are relatively large (e.g. the loss of 2 i.u./g. in a sample containing 15 i.u./g. is much more serious than the same loss from a sample with a potency of 150 i.u./g.). It seems justifiable to assume that an estimate of the content of vitamin A alcohol obtained indirectly by subtracting the potency of the ester fraction from the total is more accurate than the direct measurement when this is less than 100 i.u./g.

The relationship between plasma levels and the concentration of unesterified vitamin in the liver is linear. This is shown in Fig. 1 (*a*) where plasma levels in i.u./100 ml. are plotted against liver alcohol in i.u./g. Fig. 1 (*b*) illustrates the much greater deviation when the same blood values are plotted against total vitamin A concentration in the livers. The points for these graphs were obtained by dividing the data into groups in which plasma values were within a limit of 10 i.u./100 ml. (e.g. between 30 and 40, 40 and 50 i.u./100 ml.). The mean plasma levels in each of these groups were plotted against the corresponding mean values for the total or alcohol concentration in the livers (see Table 1).

In Fig. 1 the curve does not pass through the origin but cuts the ordinate at a point representing a plasma level of 35–40 i.u./100 ml. Thus at zero liver storage the plasma level is still 35–40 i.u./100 ml. indicating that physiological needs must be satisfied

Table 1. *The relation between vitamin A plasma levels and free and total liver levels in rats*

Vitamin A administered (i.u.)	Time after dosing (days)	Weight of Rat (g.)	Weight of Liver (g.)	Vitamin A in plasma (i.u./100 ml.) Individual value	Vitamin A in plasma (i.u./100 ml.) Mean value for group	Vitamin A in liver (i.u./g.) Total	Vitamin A in liver (i.u./g.) Alcohol Individual value	Vitamin A in liver (i.u./g.) Alcohol Mean value for group	Alcohol (%)	Ester (%)
Adult male rats:										
0	—	—	7·96	38		71	13			
10,000	5	241	9·03	36	35	307	22	25	11	89
10,000	5	183	6·88	31		285	40			
0	—	—	8·42	46		278	52			
10,000	4	224	9·00	44	45	372	64	58	18	82
50,000	3	219	7·56	57		636	61			
50,000	4	215	7·36	55	56	3,605	135	125	6	94
50,000	7	206	8·48	57		2,020	180			
150,000	5	206	6·19	67	67	4,030	145	145	4	96
500,000	10	210	6·30	75		8,190	175			
150,000	6	202	7·55	75	75	5,360	280	228	3	97
1,000,000	7	145	6·67	86		16,580	271			
100,000*	4	553	19·85	90	88	3,270	243	257	3	97
1,000,000	6	150	5·85	112	112	10,510	360	360	3	97
150,000	7	170	5·67	125		6,130	500			
500,000†	6	—	44·62	124	125	7,400	549	525	8	92
500,000*	14	553	20·95	147	147	13,500	618	618	5	95
500,000	6	170	5·59	183	183	11,040	820	820	7	93
6 young rats:										
0	—	348	17·62	50	50	15	—	⩽15	—	—
8 young rats:										
Depleted of vitamin for 7 days	—	—	18·04	47	47	4	2·6	2·6	65	35

* Groups of 3 rats. † Groups of 6 rats.

by maintenance at this level before liver storage occurs. This confirms the findings of Lewis *et al.* (1942). Even more direct evidence was supplied by two experiments carried out on almost depleted young rats (B in Table 2) in which it was found that the blood values were maintained at 47 and 50 i.u./100 ml. respectively, even when liver stores were almost nil. In these cases, accurate estimations of free vitamin A were difficult for livers of such low potency, but it was proved that a considerable proportion of the vitamin A in the livers of the rats existed in the free form.

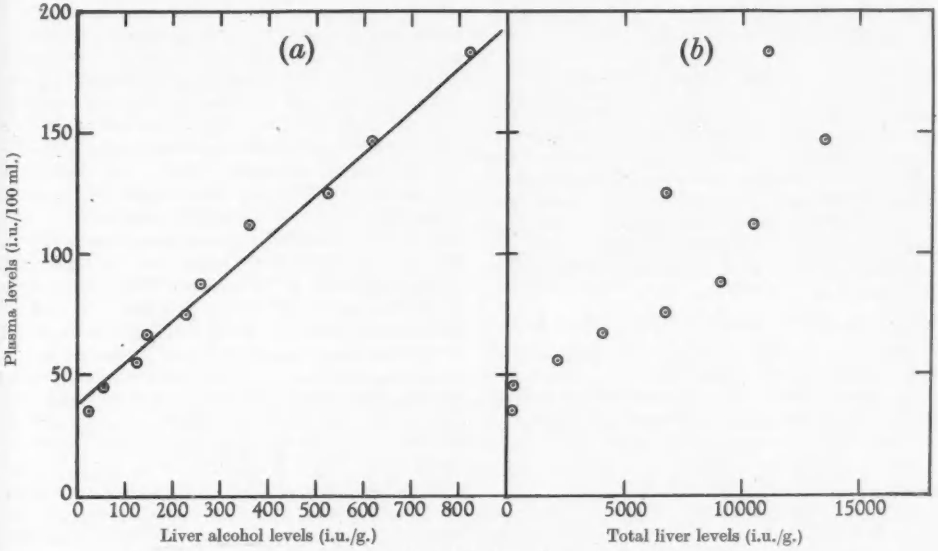

Fig. 1. (*a*), relation between vitamin A plasma levels and liver alcohol levels. (*b*), relation between vitamin A plasma levels and total liver levels.

Rate of absorption of large doses of vitamin A

In this part of the work it was essential that the plasma samples examined should be free from vitamin A ester in transit. Ester is present during active absorption, for the free vitamin A liberated in the alimentary tract by intestinal enzymes is re-synthesized to ester during passage through the gut wall (Gray & Cawley, 1942) and it is this esterified vitamin A in transport to the liver which, superimposed on the normal vitamin A alcohol content of the plasma, gives rise to the typical tolerance curve.

The time required therefore to complete the absorption of vitamin A supplements had to be determined. A pooled sample of plasma from rats killed 4 days after dosing each with 100,000 i.u. was examined chromatographically and the presence of vitamin A ester could not be demonstrated. It seems that absorption is complete within 4 days and all subsequent tests except one were carried out on rats kept for at least 4 days after dosing. The exception, in which a rat kept only 3 days was used, appears in retrospect entirely normal.

Although rats were killed not less than 12 hr. after feeding, there was also a possibility that absorption of small amounts of vitamin A from the stock diet might still be taking place. No vitamin A ester could be demonstrated in the plasma of normal animals killed 12 hr. after feeding.

Table 2. *Effect of autolysis on vitamin A content of liver tissue*

Conditions of storage	Vitamin A (i.u./g. liver) Total	Free
A. Fresh	181	35
24 hr. at room temp.	173	46
B. Fresh	71	14
72 hr. in ice-chest	70	10
C. Fresh	372	66
24 hr. at room temp.	348	57
D. Fresh	307	21
48 hr. at room temp.	291	14
E. Fresh	631	58
24 hr. at room temp.	578	74

Autolytic processes which set in immediately after removal of the liver could conceivably cause

hydrolysis of the esterified vitamin A. If this occurred to any appreciable extent, delay in examining the livers could lead to very misleading results. A number of experiments were carried out to determine the extent of hydrolysis of esterified vitamin A under varying conditions and times of storage. The results obtained (Table 2) prove that rat livers may be kept at room temperature for some hours before examination, and even 48 hr. standing caused losses no greater than 5% and did not alter appreciably the ester/alcohol ratio.

DISCUSSION

Our results make it probable that the plasma vitamin A level of rats is maintained by, and is proportional to, the free vitamin A concentration in the liver. The underlying mechanism remains obscure but it obviously tends to maintain the vitamin A *status quo* of the blood. Normal plasma values for rats vary between 35 and 50 i.u./100 ml. and in order to maintain such levels after large doses of vitamin A the equilibrium,

vitamin A ester $\rightleftharpoons$ vitamin A alcohol + fatty acid,

moves far to the left. Under reversed conditions, when liver stores are almost exhausted, the equilibrium is displaced to the right so as to maintain the normal concentration of functional vitamin A (alcohol) in the plasma, e.g. total liver store 13,500 i.u./g., % free vitamin A 5%; total liver store 4 i.u./g., % free vitamin A 65%.

The recent work of Sobotka, Kann & Winternitz (1944) on the differences in fluorescence exhibited by vitamin A esters and the free alcohol provides an explanation of the results obtained by Popper & Brenner (1942) on the fluorescence of vitamin A in the liver cells. The cytoplasmic fluorescence typical of vitamin A alcohol fades quickly, whereas the fluorescence of lipid droplets rises to a peak after a few seconds and then gradually fades; this behaviour is characteristic of esterified vitamin A. The implications of the above interpretation are that the vitamin A alcohol is dispersed in an aqueous medium (probably as a protein complex) whilst the ester occurs in the fat droplets; furthermore, an esterase converting the ester to alcohol might be expected to function only at an oil-water interface. The amount of ester brought into contact with the enzyme would then be directly proportional to the area of this interface; the vitamin A concentration in the droplets would be less important. The amount of interaction is thus probably determined by the surface area of the droplets since by alteration of size or number of droplets present, the amount of vitamin A ester exposed to the enzyme could be raised considerably without alteration in the concentration of vitamin A in the droplets themselves. The further observation of Popper & Brenner (1942), that as rats are depleted of vitamin A the droplets in the liver which fluoresce become smaller and more numerous, fits well. To convert more ester into alcohol, more ester is exposed to the enzyme by an increase in interfacial area (more droplets). Recent reports suggest that, although normal therapeutic doses of vitamin A are ineffective in improving the health of patients with no history of vitamin A deprivation, massive doses can confer considerable clinical benefits (e.g. Dunlap & Loken, 1942; Straumfjord, 1942). Our observations offer a possible explanation. An increase in the supply of vitamin A to the various organs of the body requires a considerable rise in functional vitamin A in the plasma. This can only be achieved by quite massive dosing.

SUMMARY

1. The plasma vitamin A levels of rats are proportional to the concentrations of vitamin A alcohol in the livers, but are not proportional to the total liver stores of vitamin A which consist mainly of esters.

2. Plasma vitamin A levels are maintained near normal (35–40 i.u./100 ml.) even when liver stores approach exhaustion.

3. Autolysis of intact liver at room temperature for 48 hr. does not result in hydrolysis of esterified vitamin A or in destruction of vitamin A to any appreciable extent.

4. The implications of the results are discussed in relation to vitamin A therapy in man.

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Determination of Certain Amino-acids by Ninhydrin Oxidation to Volatile Aldehydes

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The quantitative determination of amino-acids in a small sample is an important aim in protein research in its present stage. Especially in the U.S.A. and in Great Britain great advances have been made in this field during recent years. In this connexion the lecture by Chibnall (1946) may be cited. Nevertheless, the elucidation of the amino-acid composition of proteins is still a difficult task and uncertainty is continuously met with in regard to the results. Investigations in which analysis should be repeated at short intervals, as is the case, for instance, when protein synthesis in plants is examined, are almost impossible to perform for technical reasons. Rapid and easily applicable methods for the determination of amino-acids are still lacking.

Oxidation of amino-acids by ninhydrin evolves carbon dioxide as noted by Ruhemann (1911) and by Grassmann & von Arnim (1934). Van Slyke & Dillon (1936, 1938) and Mason (1937, 1938) found that the carboxyl group is thereby split off quantitatively. On the basis of this finding they developed a manometric method for the quantitative determination of the carboxyl group in amino-acids.

In this laboratory we have examined the aldehydes which are formed as the other oxidation product in the ninhydrin oxidation of amino-acids. Already before the war we had considered the possibility of determining aliphatic monoamino-monocarboxylic acids by means of volatile aldehydes ($R.CH(NH_2).COOH \rightarrow R.CHO$) formed through the action of ninhydrin. It was found that alanine, valine, leucine and its isomers, phenylalanine and methionine quantitatively form the corresponding aldehydes (acetaldehyde, *iso*butylaldehyde, *iso*-valeraldehyde, methylethylaldehyde, phenylaldehyde and methylethylaldehyde, phenylaldehyde and methylthiopropionaldehyde) which, being volatile, can easily be separated from the reaction mixture and fixed with bisulphite solution. The method based on this principle was developed for determination of the said group of amino-acids (Virtanen & Laine, 1938*a*, *b*; Virtanen, Laine & Toivonen, 1940), which is otherwise difficult to determine.

We have now re-examined this method, developed further its accuracy and removed one source of error. The method in its present form is described below (cf. Virtanen & Rautanen, 1946).

EXPERIMENTAL

Previously we determined the said group of amino-acids (then named 'aldehyde-amino-acids', a more appropriate name is 'volatile aldehyde amino-acids') by means of steam distillation whereby the aldehydes were collected in bisulphite solution in a U-tube connected to the end of the condenser. As some bisulphite-fixing substance is also formed from aspartic acid, we first removed aminodicarboxylic acids from the protein hydrolysate by a Foreman precipitation, and only after that carried out the ninhydrin oxidation.

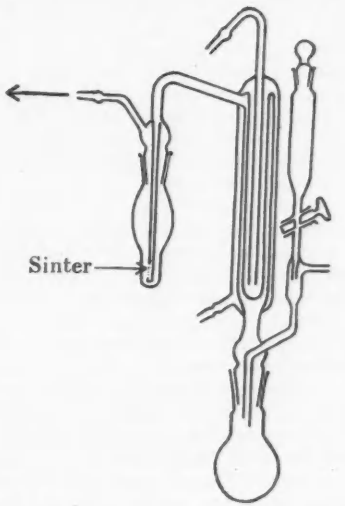


Fig. 1. Lieb-Zacherl apparatus used in determinations of volatile aldehydes formed from amino-acids.

In further development of the method we have used, instead of steam distillation, the apparatus devised by Lieb & Zacherl (1932) for lactic acid determination (Fig. 1). Volatile aldehydes are thereby sucked by a current of air from the reaction flask into the receiver and the evaporation of water vapour is prevented to a great extent by effective cooling. In the previous papers only acetaldehyde arising from alanine was determined in this apparatus, since its separation from higher aldehydes formed from other amino-acids was thought possible. This is not the case, however. According to our later determinations the separation of

acetaldehyde from other aldehydes is not possible by this method. On the contrary, during a longer reaction time all the aldehydes concerned are quantitatively trapped in the bisulphite solution in the receiver. All 'volatile aldehyde amino-acids' can thus be determined in the said apparatus.

The use of the Lieb-Zacherl apparatus for the determination of 'volatile aldehyde amino-acids' has certain advantages over steam distillation, viz. (i) it is not necessary to remove aspartic acid before the analysis; (ii) acetaldehyde and hence alanine can be separately determined (for instance colorimetrically) in the bisulphite solution, the volume of which remains approximately constant. With steam distillation, on the other hand, the volume of bisulphite solution is considerably increased, making it more difficult to determine acetaldehyde separately.

When 'volatile aldehyde amino-acids' are determined in the Lieb-Zacherl apparatus, aldehydes formed from phenylalanine and especially from methionine are distilled slowly and define the distillation time, which depends also on the vigour of the air stream, on the effectiveness of heating and on the length of the condenser tube. Other than 'volatile aldehyde amino-acids' do not give aldehydes which are distilled over into the bisulphite solution, at least not to such an extent as to have any noteworthy effect on the results (cf. the data below).

METHOD

Two apparatuses were used; the length of the condenser tube was 10 cm. in the one and 12 cm. in the other. The temperature of the cooling water was from 6 to 8°; the flame was adjusted so that boiling was rather vigorous.

Ten ml. of the solution to be investigated, the monoamino-acid content of which corresponds to about 0·05–0·2 mg. N, are placed in the reaction flask of the Lieb-Zacherl apparatus, and 1 g. of KH_2PO_4 and 2·1 g. of NaCl are added. Ten ml. of 1 % sodium bisulphite are placed in the receiver. A strong stream of air is sucked through the apparatus, and the solution is brought to the boil over a small flame. Ninhydrin solution (2 ml., the required concentration of ninhydrin is given below) is let from the side tube into the solution, whereupon the level of the surface of the solution is marked with grease pencil on the side of the reaction flask. Distillation is continued for 75 min., after which the heating is interrupted and the receiver disconnected. The tube with sintered end is washed with water and the bisulphite solution titrated with 0·01 N-iodine solution.

Distillation cannot be continued beyond 75 min. with the same lot of bisulphite solution since sulphur dioxide is removed gradually from the bisulphite solution by the air current. A new lot of bisulphite solution is then placed in the receiver, and the reaction flask made up to the original volume with water via the side tube, after which the operation is repeated for a further 75 min. without further ninhydrin addition. If methionine is present among the amino-acids to be investigated the operation must be repeated a third time for 75 min. After this all volatile aldehydes are removed with certainty. When three successive distillations of 75 min. are used, variations in the vigour of the air stream and in the size of flame are not nearly so important as when shorter distillation times are used, since in the former case the aldehydes have time to distil over more quantitatively.

In solutions which contain both amino-acids yielding volatile and non-volatile aldehydes, the amount of aldehydes distilled into the bisulphite solution during three periods of 75 min. corresponded to 99–101 % of the 'volatile aldehyde amino-acids' only. When distillation is continued after this, the additional iodine consumption of the bisulphite solution is no greater than in a blank; this shows that aldehydes formed from other amino-acids are not distilled over. Before this method is applied to an unknown solution, it is important to perform a test with a protein hydrolysate of known composition, in analysis with a known mixture of amino-acids in order to find out the appropriate velocity of the air stream and the size of flame.

RESULTS

The analyses in Table 1 illustrate the accuracy of the method.

Analysis of casein hydrolysate. The application of our method to analysis of casein hydrolysate is illustrated by the four experiments recorded in Table 2.

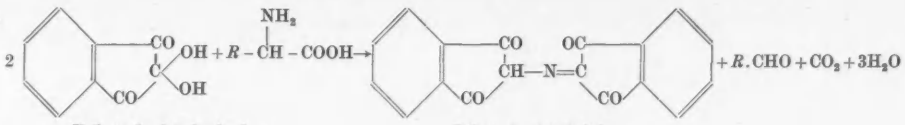
According to the latest determinations made in the laboratory of Chibnall (1946) the following amounts of the amino-acids in question were found:

	Casein N as % protein-N
Alanine	3·2
Valine	4·6
Leucine	7·2
*Iso*leucine	3·6
Phenylalanine	3·5
Methionine	2·1
Total	24·2

The agreement between our values (average 24·6) and those of Chibnall (24·2) is good.

The amount of ninhydrin necessary

As the ninhydrin is an expensive substance we examined more closely with how small an amount of ninhydrin reliable results could still be obtained. If the reaction between ninhydrin and α-amino-acid occurs in the way Ruhemann (1911) assumes (see formula on p. 977), the ratio of ninhydrin to amino-N would be 356/14, or 25·4. Thus 0·1 mg. amino-N would require 2·54 mg. ninhydrin.



Triketo-hydrindenhydrate (Ninhydrin)

Diketo-hydrindylidendiketo-hydrindamine

Our experiments on the effect of varying amounts of ninhydrin upon the results are illustrated by the following examples. The composition of the reaction solution and the experimental conditions were the same as above in all experiments except that the amount of ninhydrin was varied.

(i) 1·0 *mg. α-alanine* + 5 *mg. ninhydrin* (*theoretical amount* 4·0 *mg.*). 2·16 ml. of 0·0096 N-iodine solution were used; amino-N found: 92·2 % of the theoretical amount. In a duplicate experiment the yield was 87·6 %.

(ii) 1·0 *mg. α-alanine* + 7·5 *mg. ninhydrin*. 2·24 ml. of 0·01 N-iodine solution were used; amino-N found: 99·7 % of the theoretical amount; a duplicate gave 98·8 %.

(iii) 1·0 *mg. α-alanine* + 10 *mg. ninhydrin*. 2·24 ml. of 0·01 N-iodine solution were used; amino-N found: 99·7 % of the theoretical value.

(iv) 1 *ml. casein hydrolysate* (= 0·474 *mg. total N containing* 0·314 *mg.* NH_2-N) + 10 *mg. ninhydrin* (*theoretical amount* 8·0 *mg.*). 1·45 ml. of 0·01 N-iodine solution were used for 1st fraction, 0·16 ml. for 2nd fraction and 0·05 ml. for 3rd fraction, total 1·66 ml. Found: 0·1162 mg. N of 'volatile aldehyde amino-acids' or 24·5 % of the total N in casein.

(v) 1 *ml. casein hydrolysate* (0·474 *mg. total N containing* 0·314 *mg.* NH_2-N) + 15 *mg. ninhydrin*. 1·46 ml. of 0·01 N-iodine solution were used for 1st fraction, 0·13 ml. for 2nd fraction and 0·06 ml. for 3rd fraction, total 1·65 ml. Found: 0·1155 mg. N of 'volatile aldehyde amino-acids' or 24·4 % of the total N in casein.

On the basis of the results above it was concluded that a quantity of ninhydrin which is approximately 50 % greater than the theoretical is sufficient for determinations as far as pure amino-acids or a casein hydrolysate are in question. In the experiments recorded in Table 1 we therefore were employing well over three times more ninhydrin than was really necessary.

If the amino-acids are to be determined in solutions which contain sugar, organic acids and other organic substances, as is the case for instance with plant extracts, the amounts of ninhydrin must be somewhat increased. This is illustrated by the following experiments:

(i) 1 *mg. α-alanine* + 20 *mg. glucose* + 7·5 *mg. ninhydrin* (*theoretical amount* 4·0 *mg.*). 2·12 ml. of 0·01 N-iodine solution were used; amino-N found: 94·3 % of the theoretical value.

(ii) 1 *mg. α-alanine* + 20 *mg. glucose* + 10 *mg. ninhydrin*. 2·24 ml. of 0·01 N-iodine solution were used; amino-N found: 99·7 % of the theoretical value.

Thus the presence of glucose increased the requirement of ninhydrin to such an extent that an excess of 150 % of ninhydrin was needed for attainment of the proper result. The presence of trichloroacetic acid in the solution also requires that more ninhydrin be used. For example:

(i) 1 *mg. α-alanine* + 100 *mg. trichloroacetic acid* + 7·5 *mg. ninhydrin*. 2·11 ml. of 0·01 N-iodine solution were used; amino-N found: 93·9 %.

(ii) 1 *mg. α-alanine* + 100 *mg. trichloroacetic acid* + 10 *mg. ninhydrin*. 2·27 ml. of 0·01 N-iodine solution were used; amino-N found: 101·0 %.

DISCUSSION

When an unknown solution is to be examined, which contains amino-acids as well as other organic substances, it is safest to carry out two parallel analyses with different quantities of ninhydrin. In this way the extent can be found to which the increased amount of ninhydrin causes a rise in the aldehydes formed.

In examining yeast and plant extracts we have removed ether-soluble substances by thorough ether extraction before determining 'volatile aldehyde amino-acids', since it has been shown that some acetaldehyde is formed from pyruvic acid under the conditions of our method. Precipitation of amino-acids from the solution would be another means of separating the amino-acids from foreign organic substances; but we have not yet tried this.

With alloxan which, like ninhydrin, also causes the formation of aldehydes from corresponding amino-acids, quantitative results have not been obtained.

In addition to the group of 'volatile aldehyde amino-acids' it is possible by our method to determine alanine separately, since acetaldehyde can be estimated in the bisulphite solution beside other aldehydes formed (cf. Roine, 1946). If phenylalanine and methionine are determined by other methods in protein hydrolysates, the aldehydes formed by them and alanine can be calculated and subtracted from the total amount of aldehydes. Thus valine + leucine + *iso*leucine are left over. We have not yet succeeded in developing a method for the separate determination of the aldehydes formed from these amino-acids.

Table 1. *Determination of 'volatile aldehyde amino-acids' separately, as groups, and in mixtures of other amino-acids*

Amino-acid	Amount taken (mg.)	Amount taken (mg. N)	Distillation time (min.)	0·01 N-iodine solution (ml.)		N found (mg.)	N found (% of theory)
Alanine	1·00	0·1574	75	2·23		0·156	99·2
Valine	1·00	0·1196	75	1·69		0·112	93·7
Leucine	1·00	0·1068	75	1·51		0·106	99·6
,,	1·00	0·1068	60	1·51		0·105	98·9
Phenylalanine	1·00	0·0848	i 75	0·94			
			ii 75	0·25	1·19	0·083	98·2
,,	0·30	0·0254	i 75	0·28			
			ii 75	0·09	0·37	0·026	102·0
Methionine	0·30	0·0281	i 75	0·17			
			ii 75	0·12			
			iii 75	0·10	0·39	0·027	97·5
Alanine	0·25						
Valine	0·25						
Leucine	0·25	0·117	i 75	1·56			
Phenylalanine	0·25		ii 75	0·10	1·66	0·116	99·4
			Exp. 1 i 75	1·92			
			ii 75	0·24			
			iii 75	0·20	2·36	0·165	98·3
Alanine	0·25		Exp. 2 i 75	1·95			
Valine	0·25		ii 75	0·27			
Leucine	0·25		iii 75	0·18	2·40	0·168	100·4
*Iso*leucine	0·25	0·167	Exp. 3 i 75	2·03			
Phenylalanine	0·25		ii 75	0·20			
Methionine	0·25		iii 75	0·16	2·39	0·167	100·0
			Exp. 4 i 75	2·04			
			ii 75	0·21			
			iii 75	0·14	2·39	0·167	100·0
Alanine	0·25						
Valine	0·25						
Leucine	0·25						
*Iso*leucine	0·25	0·154	Exp. 1 i 75	1·83			
Phenylalanine	0·25		ii 75	0·26			
Methionine	0·10		iii 75	0·11	2·20	0·154	100·2
Serine	0·20		Exp. 2 i 75	1·96			
Lysine	0·10		ii 75	0·21			
Arginine	0·10		iii 75	0·07	2·24	0·157	101·7
Histidine	0·10		Exp. 3 i 75	1·88			
Proline	0·15		ii 75	0·21			
Oxyproline	0·10		iii 75	0·09	2·18	0·153	99·3
Tryptophane	0·10		Exp. 4 i 75	1·95			
Cystine	0·10		ii 75	0·20			
Tyrosine	0·10		iii 75	0·08	2·23	0·156	101·3
Glutamic acid	0·35						
Aspartic acid	0·10						

1% (w/v) ninhydrin solution (2·0 ml.) was used in all experiments except the last, in which the solution was 2%.

Table 2. *Replicate determinations of 'volatile aldehyde amino-acids' in casein hydrolysate*

(1 g. casein (=142·2 mg. N) was hydrolyzed with 10 ml. of conc. HCl for 6 hr. The hydrolysate + 100 ml. of water was evaporated *in vacuo* to a syrup. The residue was dissolved in water and made to 200 ml. A sample of 0·5 ml. (=0·355 mg. N) was used for one determination.)

No. of analysis	1st distillation 75 min. (0·01 N-iodine solution (ml.))	2nd distillation 75 min. (0·01 N-iodine solution (ml.))	3rd distillation 75 min. (0·01 N-iodine solution (ml.))	Total (0·01 N-iodine solution (ml.))	N found (mg.)	N found (% protein-N)
1	1·00	0·15	0·08	1·23	0·0861	24·3
2	1·11	0·10	0·06	1·27	0·0889	25·0
3	1·02	0·17	0·07	1·26	0·0882	24·8
4	1·03	0·15	0·05	1·23	0·0861	24·3
					Average	24·6

SUMMARY

1. A method based on the quantitative formation of volatile aldehydes from α-alanine, valine, leucine, *iso*leucine and possibly other isomers, phenylalanine and methionine ($R.CH(NH_2).COOH \rightarrow R.CHO$) has been used for the quantitative micro-determination of these amino-acids as a group ('volatile aldehyde amino-acids'). With other amino-acids found in proteins no formation of volatile aldehydes was detected under our experimental conditions.

2. Acetaldehyde can be determined separately from the mixture of aldehydes. Thus it is possible to determine by means of volatile aldehydes (*a*) the above group of amino-acids, and (*b*) alanine. If phenylalanine and methionine are determined separately by specific methods, valine, leucine and *iso*leucine remain to be determined. Separate determination of the aldehydes formed from them has not yet been successful.

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Experiments on the Localization and Nature of Tea Oxidase

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The conversion of green tea leaf into the product known as black tea involves the oxidation by leaf enzymes of the phenolic tannins of the leaf, the oxidation being initiated by the crushing of the wilted leaf. Ever since the recognition of the tea fermentation (as the process is generally known) as an enzymic oxidation, attempts have been made to identify the enzyme involved with one or another of the known oxidases. Early workers, notably Mann (1901), Bernard & Welter (1911) and Manskaya (1935) have suggested identity of the tea fermentation enzyme with peroxidase, while tyrosinase (Potapov, 1934) and ascorbic acid oxidase have also been considered (Roberts, 1939*a*, *b*). Recent investigations carried on by Lamb & Sreerangachar (1940) in Ceylon and by Roberts and Roberts & Sarma (1938–42) in India have shown, however, that the tea enzyme is neither peroxidase nor ascorbic acid oxidase. Thus the simultaneous presence in the tea leaf of both peroxidase and the tea tannin does not suffice for fermentation. Ascorbic acid oxidase, if it is indeed present in the leaf at all, does not participate in the fermentation either directly or indirectly. According to Roberts (1942) tea leaf contains a cytochrome oxidase which is capable of oxidizing (presumably through the intermediary of cytochrome) tea tannin, catechol and *p*-phenylenediamine. The workers in India further believe that this same oxidase is the terminal oxidase of the normal respiration of the tea leaf. The views of Roberts and his group (1938–42) are mainly based on experiments carried out with whole minced leaves and these crude enzyme preparations always contained the tea tannin so that substrate specificity of the enzyme could not be clearly studied. The Ceylon workers, on the other hand, have shown (Lamb & Sreerangachar, 1940; Sreerangachar, 1941, 1943*a*, *b*, *c*, *d*) that the tea enzyme is a polyphenol

oxidase which is highly specific and which oxidizes *o*-polyphenols. Their work was done with purified enzyme preparations free of endogenous substrate. Recent work of Sreerangachar has also shown that the tea oxidase as it exists in the leaf is insoluble and appears to be associated with the solid portion of the cell. Some soluble oxidase activity could, however, be obtained by repeated extraction with buffer of acetone-extracted dried ground leaves. The soluble enzyme contains copper and the activity of purified preparations is proportional to their copper content (Sreerangachar, 1943*d*).

The present work is concerned with a further study of the nature of the tea oxidase, the location of the enzyme within the cell, and finally the relation of tea oxidase to the respiration of the tea leaf.

EXPERIMENTAL

Materials and methods

Plants of the Chinese tea, *Thea Sinensis* var. *Bohea*, were used as a source of leaves for these investigations. The plants were obtained from a nursery where they had been grown from seed collected from an unknown source, probably Japan. They were grown in 5 gal. cans maintained under greenhouse conditions and grew vigorously throughout.

The bud and first two leaves of the new growth were used as a source of enzyme for all experiments unless otherwise stated. This material corresponds to that used in the preparation of ordinary teas. The leaves were ground in phosphate buffer and enzyme preparations made in a variety of ways detailed below. All operations were carried out at 0° in order to minimize inactivation of the enzyme.

Oxidase activity was determined by the measurement of oxygen uptake in the Warburg respirometer. The enzyme preparation was suspended in 2 ml. of 0·1 M-phosphate at pH 5·5 in conical vessels having a total vol. of approximately 16 ml. The central well of each vessel contained 0·3 ml. of 5% KOH, while 0·3 ml. of substrate was added from the side-arm of the vessel after attainment of temperature equilibrium. A reaction temperature of 30° was used throughout.

Preparation of the active enzyme

That tea leaves contain an active oxidase may be shown as follows:

Fresh tea leaves were ground by hand in a mortar under 0·1 M-phosphate buffer (pH 6·7), filtered through cheese-cloth to remove cellular debris and the filtrate centrifuged at high speed for 30 min. The clear yellowish supernatant liquid contains chiefly the tea tannins, sugars, and soluble nitrogenous compounds including the cytoplasmic proteins, and exerts little enzymic activity toward catechol (see below). The precipitate from the centrifugation was washed with buffer, recentrifuged, and this repeated four times to remove completely the tea tannins. Microscopic observation showed that this preparation consisted largely of chloroplasts and of chloroplastic material, especially the constituent grana or green particles of which the plastid is formed. Small amounts of cell wall material, crystals of calcium oxalate, and leaf hairs were also present.

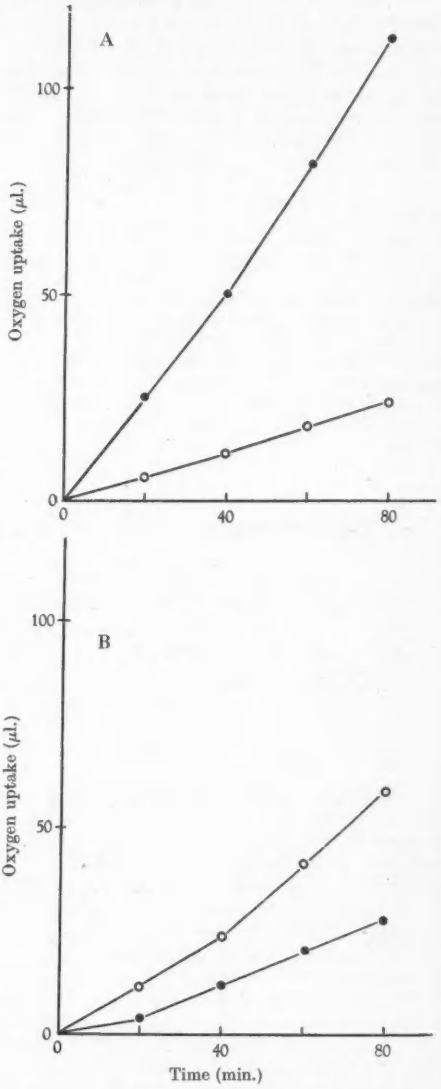

Fig. 1. Rate of oxidation of catechol and of *p*-phenylenediamine by preparations from tea leaves. A. Oxidation of catechol by the insoluble (upper curve) and the soluble (lower curve) portions of ground tea leaves. B. Oxidation of *p*-phenylenediamine by the soluble (upper curve) and insoluble (lower curve) portion of ground tea leaves.

From the standpoint of the leaf proteins, the purified precipitate represents what has been termed by Chibnall (1939) the chloroplastic fraction, while the cytoplasmic proteins are present in the original supernatant liquid. Samples of each fraction were tested for activity in the oxidation of catechol and of *p*-phenylenediamine. It may be seen (Fig. 1) that the chloroplast-grana fraction rapidly oxidizes catechol but has little effect on *p*-phenylenediamine, while with the cytoplasmic fraction just the reverse is true. It might at first be thought that two kinds of enzyme systems are here involved, namely a polyphenol-like oxidase located in the chloroplasts, and *p*-phenylenediamine oxidase or oxidase system located in the cytoplasm. It can be shown, however, that the oxidation of *p*-phenylenediamine is non-enzymic and persists even with cytoplasmic preparations which have been heated to 100° (Table 1).

Table 1. *Non-enzymic nature of p-phenylenediamine oxidation by the soluble fraction of tea leaf*

Substrate	O_2 consumption (μl./vessel/60 min.) Untreated preparation	Boiled preparation
p-Phenylenediamine	83·1	94·0
Catechol	2·6	0·0

It is possible that the oxidation of *p*-phenylenediamine by tea leaves as described by Roberts (1941 *b*) and as observed here represents merely a non-enzymic reaction between the tea tannins or other oxidizing agents and the *p*-phenylenediamine.

Repeated attempts were made to obtain a soluble oxidase from the supernatant liquid by precipitation, on the supposition that the presence of such an enzyme might be masked due to inactivation by the tea tannins. Precipitation with ammonium sulphate or with acetone failed to yield any fraction active in oxidizing catechol or *p*-phenylenediamine. It must be concluded, therefore, that the tea oxidase is not present in the cytoplasmic fraction, but is present rather in the chloroplastic fraction. That the oxidase is present in the chloroplast itself is strongly indicated by the experiment of the following section.

Oxidase activity of intact plastids

In order to establish the association of oxidase activity with the chloroplasts, a procedure for obtaining intact chloroplasts similar to that described by Granick (1938) was used. In this procedure the leaves are ground in an isotonic sugar solution in order to avoid the bursting of the plastids which occurs in hypotonic solutions.

Fresh tea leaves were ground under a 10% (w/w) glucose solution for 90 sec. in a blender. The resultant *brei* was filtered through cheese-cloth to remove unground leaf fragments and cell walls, after which the filtrate was centrifuged (2000 r.p.m. in an International clinical centrifuge). The precipitate which contained, in addition to intact chloroplasts, a few grana from broken plastids, calcium oxalate crystals, etc., was again taken up in 10% glucose and recentrifuged at low speed (approximately 500 r.p.m. in an International clinical centrifuge). At this low speed the large plastids could be separated from the grana and other smaller particles.

Repetition of this procedure five times resulted in a preparation which microscopic examination revealed to consist exclusively of intact chloroplasts. Such chloroplasts actively oxidize catechol, as is shown in Table 2. Furthermore, the enzymic

Table 2. *Oxidase activity of intact chloroplasts of tea leaves together with the activity of grana derived from such chloroplasts*

	O_2 uptake (μl./$\frac{1}{2}$ hr./mg. enzyme) at $\frac{1}{2}$ hr.	1 hr.	1$\frac{1}{2}$ hr.	2 hr.
Original leaf extract in 10% (w/w) glucose solution	1·21	1·09	0·80	0·80
Purified intact plastids	2·17	3·90	3·90	4·33
Plastids ground with quartz sand (granas)	4·09	3·87	3·44	3·01
Supernatant granas from purified intact plastids	1·98	2·10	1·73	1·48
Hand-ground leaf extract	6·46	5·70	4·37	3·80
Acetone-purified hand-ground leaf extract	7·29	7·02	5·67	4·86

activity of the pure intact chloroplast preparation is greater on a dry weight basis than the activity of the original whole precipitate, including chloroplasts, grana and other cellular material. It would appear probable, therefore, that the catechol oxidase activity is actually associated with the chloroplast rather than with some other kind of cellular particle.

Many methods of obtaining grana from intact chloroplasts were attempted. The most successful consisted in grinding the chloroplast preparation with quartz sand under buffer. The quartz sand was then centrifuged from the grana suspension by low-speed centrifugation. Table 2 shows that when the chloroplasts are so treated the resultant grana have an oxidase activity toward catechol even greater than that of the original chloroplast. In addition, the gradual increase in reaction rate with time, observed with intact chloroplasts, is not observed with the grana preparation. Whether the permeability of the intact plastid to catechol gradually increases with time or whether the lag in attainment of maximum rate merely represents the time needed for inward diffusion of the substrate is not revealed by these experiments. Table 2 includes for comparison a

preparation made by grinding of leaves by hand in a mortar. In this case, which is similar to that recorded in the experiment of Fig. 1, the insoluble enzyme fraction consists mainly of chloroplastic grana. The oxidase activity of the preparation is comparable to that of the grana obtained from intact plastids.

It can be concluded then that an active insoluble oxidase is contained in the chloroplasts of tea leaves and that this enzyme is associated with the grana.

The following experiment indicates, however, that the enzyme is not directly connected with the chlorophyll of the grana. A purified grana preparation was extracted with aqueous 40 % (v/v) acetone. This treatment removed the bulk of the chlorophyll from the grana. The residual particles possessed as great or greater oxidase activity than the original intact grana (Table 3). This observation is in agreement with that made by Lamb & Sreerangachar (1940) who prepared the tea oxidase by a procedure involving a preliminary grinding of the leaf in aqueous acetone.

Table 3. *Effect of acetone extraction of tea oxidase on activity toward catechol*

(Each vessel contained 3 mg. catechol.)

	O_2 consumed (μl./hr./vessel)	
	Exp. 1	*Exp.* 2
Enzyme preparation	29·5	32·0
Same preparation after extraction with 40 % (v/v) acetone	37·0	41·2

It has not been possible to break down the grana further and to release quantitatively the oxidase in soluble form. It has, however, been possible to obtain small amounts of soluble oxidase by repeated extraction of dried grana as is shown in Table 4.

Table 4. *Conversion of the insoluble tea oxidase into soluble form by drying* in vacuo

(All determinations made in 0·1 M-phosphate, pH 5·5, with 3 mg. catechol/vessel.)

Preparation	O_2 consumed (μl./hr./10 mg. original enzyme)
10 mg. of whole dried acetone-extracted grana	32
1st extract of 10 mg. grana (extracted 3 hr. in buffer)	30
2nd extract (3 hr.)	18
3rd extract (19 hr.)	18
Residue after 3 extractions	33

Grana preparations were taken to dryness in a vacuum desiccator at room temperature and were then extracted with repeated portions of 0·1 M-phosphate, pH 5·5. As can be seen in Table 4, only a small amount of the total oxidase was extracted in soluble form since the residual particles after extractions were still highly active.

Substrate specificity of the tea oxidase

The insoluble enzyme contained in the grana of tea leaves attacks a wide variety of phenols, including the tea tannin (hot water extract from fresh tea leaves), catechol, gallic acid and dihydroxyphenylalanine. It fails to attack monophenols such as *o*- and *p*-cresol and tyrosine. It does not attack *p*-phenylenediamine at any considerable rate.

Table 5. *Specificity of tea oxidase*

(Acetone extracted, vacuum dried crude grana as enzyme. Concentration of all substrates: 1·5 mg./ml.)

Substrate	O_2 consumption (μl./10 mg. enzyme/hr.)
None	4·4
Catechol	72·5
Phloroglucinol	7·7
Dihydroxyphenylalanine	44·3
o-Cresol	5·3
p-Phenylenediamine	10·8
p-Cresol	0·0
Gallic acid	17·4
p-Hydroxybenzoic acid	4·3
Tyrosine	0·0

Inhibition of tea oxidase

The action of the tea oxidase on catechol is relatively insensitive to cyanide, 0·001 M-KCN exerting but little effect on the rate of the oxidation. Tea oxidase is inhibited by low concentrations of *p*-nitrophenol, a substance which has been shown to inhibit the polyphenol oxidase of spinach leaves (Bonner & Wildman, 1946); 1 mg./ml. of this substance caused 86 % inhibition in the rate of oxidation of catechol, while one-tenth of this concentration still brought about a significant depression in rate (Table 6).

Table 6. *Effect of various inhibitors on tea oxidase*

(Substrate, 3 mg. catechol/vessel in all cases. 10 mg. acetone-extracted whole grana/vessel.)

Inhibitor	O_2 consumption (μl./10 mg. enzyme/hr.)	Inhibition (%)
None	39·9	—
KCN, 0·01 M	7·1	82·3
KCN, 0·001 M	38·1	4·5
None	72·8	—
p-Nitrophenol: 1 mg./ml.	19·5	73·0
p-Nitrophenol: 0·1 mg./ml.	42·6	41·0

Relation of tea oxidase to normal leaf respiration

The respiration of excised tea leaves was studied with preparations which had been chopped into fragments approximately 4 sq.mm. in area. The leaf

fragments (0·1 g.) were then weighed into each Warburg vessel, 2 ml. of 0·1 M-KH_2PO_4, pH 4·5 added, and gas exchange studied in the usual manner. The penetration of added substrates or inhibitors into the leaf was assured by twice repeated vacuum infiltration of the leaf fragments in the test solution. Added catechol was without significant effect on O_2 uptake or the CO_2 evolution of tea leaves (Table 7). This behaviour is in marked

Table 7. *Effect of catechol on the respiration of tea leaves*

(Leaves vacuum-infiltrated with catechol; 100 mg. leaf fragments (*c.* 4 sq.mm.) in 2·0 ml. 0·1 M-KH_2PO_4, pH 4·5.)

Substrate	Gas	Gas exchange (μl./100 mg. leaf/hr.) *Exp.* 331	*Exp.* 330
None	O_2	77	58
	CO_2	69	53
Catechol (1 mg./ml.)	O_2	80	57
	CO_2	70	60

contrast to that of spinach leaves in which polyphenol oxidase is the terminal respiratory oxidase (Bonner & Wildman, 1946). These rapidly oxidize catechol, while at the same time CO_2 evolution by the leaf is greatly depressed. It would appear that in tea leaves, on the contrary, added catechol does not penetrate to the site of the tea oxidase, and it is unlikely that this oxidase participates in respiration, particularly since, as has also been shown in the work with spinach leaves, other respiratory enzymes such as the dehydrogenases probably occur among the cytoplasmic proteins. Potassium cyanide in the concentration of 0·001 M, which has been shown above to be without effect on the tea oxidase in its action on catechol, has some inhibiting effect on normal tea-leaf respiration; *p*-nitrophenol, on the other hand, is less inhibitory to respiration than to the tea oxidase (Table 8). These facts taken in conjunction make it appear improbable that the tea oxidase participates in the normal respiration of tea leaves.

Table 8. *Effect of inhibitors on the rate of respiration of tea leaves*

(Inhibitors infiltrated into leaves suspended in 0·1 M-KH_2PO_4, pH 4·5.)

Addition	O_2 uptake (μl./100 mg. leaf/hr.) 1st hr.	2nd hr.	Inhibition in 2nd hr. (%)
None	72	66	—
KCN, 0·001 M	49	43	35
p-Nitrophenol: 1 mg./ml.	50	38	42
p-Nitrophenol: 0·1 mg./ml.	67	64	3

DISCUSSION

The enzyme responsible for the oxidation of the tea tannins and the fermentation of black tea resembles polyphenol oxidase in that it can carry on direct oxidation of catechol and related *o*-diphenols. The polyphenol oxidases which have been studied extensively, such as those of the potato tuber (Kubowitz, 1937), mushrooms (Graubard & Nelson, 1935), and spinach leaves (Bonner & Wildman, 1946), are all readily soluble proteins occurring in the cytoplasm of the plant, and in spinach leaves polyphenol oxidase is definitely absent from the chloroplasts. In tea leaves, on the contrary, the oxidase appears to be normally absent from the cytoplasm and to occur solely in the chloroplasts. The enzyme does not depend on the chlorophyll, since removal of this material with acetone leaves the oxidase activity of the plastid or grana undiminished. The enzyme is however closely associated with the structure of the grana, and it has not been possible quantitatively to extract or remove the oxidase from the grana. This fact accounts for the insolubility of the enzyme, reported by earlier workers. Tea oxidase is but one of a series of enzymes (e.g. succinic dehydrogenase, cytochrome oxidase, etc.), whose activity is associated with the structure of the cell. In the present case, however, insolubility of the enzyme can be attributed to the anatomic aspects of its distribution within the cell. The enzymic activities of isolated chloroplasts and of grana have been but meagrely investigated. Neish (1939) has reported that isolated grana of *Trifolium* and other chloroplasts exhibit catalase and carbonic anhydrase activity.

Polyphenol oxidase has been shown to be the terminal oxidase in the respiration of the potato tuber (Baker & Nelson, 1943; Boswell & Whiting, 1938) and in the spinach leaf (Bonner & Wildman, 1946). In tea, however, it would appear that the normal leaf respiration does not involve polyphenol oxidase since respiration remains unaffected either by inhibitors of the enzyme or by the addition to the leaf of substrates such as catechol. The normal leaf respiration of tea must therefore involve other and as yet unrecognized oxidase systems. Clearly also the tea tannins which are present in the intact cell do not normally come in contact with the tea oxidase. Contact between tannin and enzyme in tea fermentation is presumably brought about by the preliminary crushing to which the leaves are subjected.

SUMMARY

1. The enzyme of tea leaves which is responsible for the oxidation of the tea tannins in tea fermentation is an oxidase having substrate specificity resembling that of polyphenol oxidase.

2. The tea oxidase, unlike the polyphenol oxidase of other tissues, is normally insoluble in water or buffer solution. The oxidase activity is associated with the chloroplasts of the leaf. If the chloroplasts are broken so as to release the grana it may be shown that the oxidase is associated with the grana.

3. Removal of chlorophyll from the grana by extraction with acetone leaves the enzyme activity of the preparation unaffected. Soluble oxidase may be extracted in small quantities from dried grana preparations.

4. The tea oxidase does not appear to participate in the normal leaf respiration of the tea plant.

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The Assay of the Vitamin B_6 Complex

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It is now known that pyridoxal, pyridoxamine and pyridoxal 'phosphate' (the co-enzyme of certain amino-acid decarboxylases) occur naturally and contribute to the 'vitamin B_6' effect by which pyridoxin was originally recognized. These compounds, closely chemically related to pyridoxin, give quantitatively very different responses with certain micro-organisms when these are used as the biological test object, while they have approximately equal potencies for the rat and the chick (Snell & Rannefeld, 1945; Luckey, Briggs, Elvehjem & Hart, 1945), and, presumably, for human beings. It is therefore convenient, if not essential, to have a microbiological assay method in which the test organism responds equally to all the compounds of the vitamin B_6 complex, so that assays of food-stuffs may be made which will give values proportional to their total content of vitamin B_6-active substances.

The yeast *Saccharomyces carlsbergensis* 4228, used by Atkin, Schultz & Frey (1943) for the microbiological determination of pyridoxin appears to fulfil this condition. Snell & Rannefeld (1945), using this yeast, found that the relative activities of pyridoxin, pyridoxal and pyridoxamine were 1·00 : 1·16 : 1·09, while Miller & Baumann (1945) found the activities equal within $\pm 5\%$. The method of assay with *S. carlsbergensis* has been used by us with slight modifications and tested for a variety of foodstuffs. The existence of an unknown member or members of the vitamin B_6 complex postulated by Melnick, Hochberg, Himes & Oser (1945) has not been confirmed by data presented here. The better-known chemical methods for the determination of vitamin B_6 have been tested and some criticisms are offered.

EXPERIMENTAL

Methods

The procedure of Atkin *et al.* (1943) was followed closely, only departures from it are stated here.

Culture. We are indebted to Dr Frey for a culture of *S. carlsbergensis* 4228. Another strain of *S. carlsbergensis* (Frohberg strain) kept in these laboratories was only stimulated slightly by pyridoxin.

Standards. Pyridoxin hydrochloride was kindly supplied by the Medical Research Council (a commercial sample was

found to possess only 92% of the activity of this); for pyridoxal, pyridoxamine and 4-pyridoxic acid we are greatly indebted to Mr R. T. Major of Messrs Merck and Co. Inc., Rahway, N.J., U.S.A.

Basal medium was as described by Atkin *et al.* (1943), ultimately modified by the addition of nicotinic acid in the same concentration as the calcium pantothenate (2·25 μg./ml.).

Method of assay. To a series of flat-bottomed specimen tubes, 10 × 1·5 cm. (internal), each containing 1·25 ml. of the medium, was added 0·2, 0·4, 0·5, 0·6, 0·8 or 1·0 ml. of the standard pyridoxin solution (0·01 μg./ml.) or 0·3, 0·6 or 0·9 ml. of the test extract calculated from a preliminary trial to contain an amount of vitamin B_6 falling within the range of the standard curve. The contents of each tube were then made up to 2·25 ml. with distilled water. Yeast suspension, 0·25 ml. (equivalent to 25 μg. of pressed yeast), was added with precautions to secure accuracy and equality of seedings. All tubes were set up in triplicate. After 16 hr. shaking in an incubator at 30° the tubes were placed for 10 min. in the refrigerator and the final suspension was diluted with 10 ml. of ice-cold water. All tubes were then shaken vigorously 20 times, and each inverted again 30 sec. before reading in a Pulfrich nephelometer or Spekker absorptiometer. If the extract to be tested was turbid, it was filtered before addition to the growth tube. Owing to the great dilution interference with the turbidimetric measurements by colour in the extracts was rarely encountered. In such cases the tubes were rapidly centrifuged after incubation, the clear coloured liquid poured off and an equal volume of dilute saline added, followed by shaking. This procedure was found reliable when tested on colourless suspensions.

Important precautions. Adequate, uniform and continuous shaking is essential and is facilitated by the use of small volumes of culture medium in the growth tubes. Temperature variation in different parts of the incubator, if allowed, is a serious source of discrepancy between triplicates.

The tubes employed for the growth tests were also used for reading in the nephelometer, having been tested for uniformity by the reading in them of standard suspensions of kieselguhr, a deviation between tubes of ±1% being allowed. For reading, the tubes were always turned into the same position. With prepared yeast suspensions nephelometer readings varied almost linearly with yeast concentration. Similar standardizations were performed for the Spekker instrument.

Improvement of the basal medium

Nicotinic acid in the same concentration as calcium pantothenate (2·25 μg./ml.) was found to increase growth by about 50% in a 16 hr. period (see Table 4), to a rate as rapid as in malt wort (15% malt), which is an ideal medium for brewers' yeasts. Asparagine, cystine, tryptophan, xanthine, choline, riboflavin, and *p*-aminobenzoic acid when added singly at levels much higher than would be present in the test extracts did not appreciably stimulate growth. It was later found that the addition to each tube of 10 mg. $(NH_4)_2HPO_4$ as a solution decreased the blank reading while slightly increasing the readings at the higher levels of pyridoxin.

Preparation of extracts

Materials were suitably sampled, finely ground if solid, and a portion containing *c.* 1 μg. of vitamin B_6 was autoclaved for 1 hr. at a pressure of 15 lb./sq. in. with 25 ml. of 2 N-H_2SO_4; after filtering and adjusting to pH 5·0 with NaOH, the volume was made up to 100 ml. It was found that a concentration of Na_2SO_4 equal to that present in the tubes when 0·9 ml. of the extracts was added did not influence the growth of the yeast. The use of 2 N-H_2SO_4 was recommended by Siegel, Melnick & Oser (1943) on the basis of results obtained with rice-bran concentrate. Increasing the time of autoclaving to 2 hr. had no significant influence, but, for a few materials, a 30 min. treatment as suggested by Siegel *et al.* (1943) gave slightly lower results. Similar treatment with 0·1 N-H_2SO_4, or the procedure of Bina, Thomas & Brown (1943) involving digestion with papain and takadiastase, gave for all materials listed in Table 3 vitamin B_6 values from 10 to 40% lower, with two exceptions mentioned below. A mixture of equal parts of commercial preparations of papain and takadiastase was found to have a vitamin B_6 content of about 2 μg./g. The above procedure did not give optimum results for yeast and dried milk. The values obtained for these materials by different extraction procedures are given in Table 2 and their significance discussed below.

With rare exceptions, which were rejected, the readings of the triplicate tubes agreed within ±7%. Vitamin B_6 values calculated for the different levels of added extract were within ±5%. Recovery of added pyridoxin was quantitative except with an extract of mixed invert sugar as used in a brewery, for which it was low, indicating the presence of inhibiting substances apparently produced by the autoclaving with acid. Hydrolysis with papain and takadiastase was used in this case; recovery was normal but the vitamin B_6 value was probably too low.

RESULTS

The figures in Table 1 confirm that the activities of pyridoxin, pyridoxal, and pyridoxamine for *S. carlsbergensis* 4228 are equal. Furthermore, the growth-time curves were identical with each compound. The very slight activity of 4-pyridoxic acid was possibly due to the presence of traces of one or more of the active compounds as impurity. The fact that the response of the organism to the known compounds possessing vitamin B_6 activity is the same as that of the rat is at present the best indication of the validity of the method. Comparison of results reported for similar materials by different methods is, for vitamin B_6, of doubtful value. Apart from inevitable variations in the materials, extraction procedures differ and the relative response to the

Table 1. *Comparative activity of pyridoxin, pyridoxal, pyridoxamine and 4-pyridoxic acid for* S. carlsbergensis 4228

Compound (0·002 and 0·004 μg./ml.)	Relative turbidity after 16 hr. growth
Pyridoxin	1·00
Pyridoxal	1·02
Pyridoxamine	0·99
4-Pyridoxic acid	0·0004

results by the *Neurospora* method, which show agreement with those obtained by the yeast method.

Yeast and dried milk gave low results when 2 N-H_2SO_4 was used for the extraction, although excellent recoveries (100 ± 7 %) of added pyridoxin, pyridoxal and pyridoxamine were secured. However, Table 2 shows that the destructive effect of 2 N-acid was not observed when the sample was first subjected to milder hydrolytic procedures. This is in

Table 2. *Apparent vitamin B_6 content of yeast and dried milk measured microbiologically after various extraction procedures*

Extraction procedure			Pyridoxin (μg./g.)		
Autoclaving with H_2SO_4 at 120°		Alternative to autoclaving	Pressed bakers' yeast (moisture 75 %)	Food yeast (moisture <10 %)	Dried milk
Concentration of acid	Time (hr.)				
0·1 N	1	—	—	20·0	—
0·1 N	2	—	6·0	24·5	4·6
0·1 N	3	—	—	26·0	—
0·1 N	5	—	—	28·6	—
2·0 N	1	—	3·4	11·4	—
2·0 N	2	—	—	12·2	3·6
0·1 N followed by 2·0 N	2 followed by 1	—	6·2	26·5	5·5
2·0 N followed by 0·1 N	1 followed by 1	—	4·9	—	4·1
None	—	Papain and takadiastase digestion at 40° for 24 hr.	—	26·7	—
None followed by 2·0 N	— followed by 1	Do.	—	26·3	—

Table 3. *The vitamin B_6 content of various products measured by the* S. carlsbergensis *assay*

Product	Vitamin B_6 content expressed as pyridoxin (μg./g.)
Flour (85 % extraction)	1·7
Dried milk	5·5
Pressed bakers' yeast	6·2
Brewers' yeast	5·7
Food yeast	28·6
Shropshire barley (13·9 % moisture)	3·2
Shropshire barley after malting (4·1 % moisture)	6·0
Malt rootlets	10·2
Mixed invert sugar from a brewery (determined after enzyme extraction)	0·14
Rose hips	1·7
	(μg./ml.)
Whitbread's ale	0·52
Whitbread's stout	0·67

components of the complex may vary between one method and another. Comparable results with identical samples have been obtained on several occasions, and a few of these are given in Tables 5 and 6.

The results in Table 5 were obtained with two samples of beer which had not been pretreated with acid. Free pyridoxin only therefore was measured. We are indebted to Dr E. Barton-Wright for the

accordance with the fact that treatment with 2 N-H_2SO_4 for 2 hr. gives values as high as, or slightly higher than, treatment for 1 hr. On the other hand, if treatment with 2 N-acid precedes the treatment with 0·1 N-acid, results are again low. These observations seem to suggest that the vitamin is destroyed more readily by 2 N-H_2SO_4 when it is in a 'bound' state. The fact that these results were obtained with yeast, which contains mainly pyridoxamine (Snell, 1945), may mean that this component is more susceptible to destruction in this way. These results also show that 100 % recoveries are not infallible criteria of the reliability of an extraction procedure.

Melnick *et al.* (1945) also obtained low values for the vitamin B_6 content of yeast after treatment with 2 N-H_2SO_4 and ascribed this to the destruction of an unknown member or members of the vitamin B_6 group. However, in view of the above results it is unnecessary to postulate their existence.

The possibility was considered that other compounds may be present in natural materials to which the organism may adapt itself in time, so as to utilize them as vitamin B_6 when suboptimal amounts of the vitamin are present. However, when the incubation time was increased from 16 to 40 hr. the results obtained with yeast, beer and dried milk were not significantly different.

Few investigations of the stability of vitamin B_6 in foodstuffs have been reported. We found no significant change in samples of beer and dried yeast during 4 months' storage in the dark at room temperature. A standard solution of vitamin B_6 (100 μg./ml.) in N/50 HCl stored in the refrigerator was unchanged in potency after 8 months.

Use of the organism for other assays

S. carlsbergensis 4228 has been used also for assaying pantothenic acid. We investigated its dependence upon four other vitamins, both in the medium used above and in one in which the casein hydrolysate was replaced by $(NH_4)_2HPO_4$ (4 mg./ml.). The results in Table 4 indicate that it may also be suitable for the assay of biotin and of inositol.

Table 4. *Response of* S. carlsbergensis 4228 *to six growth factors*

One growth factor at a time was omitted from the group of six named in column 1.

	Nitrogen source	
	Casein hydrolysate	$(NH_4)_2HPO_4$
Growth factors in medium	Yield of yeast, dry weight: mg./tube (2·5 ml.)	
All six	3·88	1·28
Inositol omitted	0·22	0·24
Pantothenic acid omitted	0·11	0·25
Nicotinic acid omitted	2·35	0·83
Biotin omitted	1·00*	0·07
Aneurin omitted	1·65	0·82
Pyridoxin omitted	0·25	0·12

* High result probably due to the presence of biotin in the casein hydrolysate.

Table 5. *Comparison of values obtained by means of methods involving the use of* S. carlsbergensis 4228 *and of* Neurospora

	Pyridoxin (μg./ml.)	
	Yeast method	*Neurospora* method
Beer, draught	0·24	0·26
"	0·25	0·28

Chemical determinations

A number of procedures described in the literature for the colorimetric method involving coupling with diazotized sulphanilic acid was found to be unsatisfactory. We found that, in addition to the variety of compounds known to couple with this reagent, biotin gives a yellow colour, which is quantitative. Bina *et al.* (1943) used alkaline ethanol elution from a 'Superfiltrol'* adsorbate to make the test specific for pyridoxin. However, we found with their procedure upon evaporating off the ethanol and carrying out the colorimetric test in aqueous solution that reddish brown interfering colours were present; the chromogenic impurities were not precipitated by aqueous $AgNO_3$ or $Ba(OH)_2$. The ethanol appears not to act altogether as a specific eluent but merely to suppress the development of interfering colours. Consequently the procedure of Bottomley (1944), involving removal of the ethanol and reaction in aqueous solution, is open to criticism. Furthermore, the method of Bina *et al.* (1943) failed to give consistent results even under fully standardized conditions with pure solutions of pyridoxin, the colour developed varying from yellow to pink. Appreciable blank readings were recorded, apparently not corrected for by these workers. Values obtained on worts and yeasts were abnormally high. The use of *p*-aminoacetophenone, α-naphthylamine, or *p*-chloraniline instead of sulphanilic acid was no more satisfactory; *p*-aminobenzoic acid was found to give the most consistent results, but only in aqueous solution. The use of a borate blank (see below) was possible only in aqueous solution.

* A proprietary adsorbent manufactured by the Permutit Co. Ltd.

The indophenol method adapted by Hochberg, Melnick & Oser (1944) by the use of a borate blank to measure only pyridoxin was satisfactorily applied to many materials, recovery of added pyridoxin being 90–100%. Modifications used by us were: (*a*) preliminary purification of the extract by addition of 2 ml. 15% sodium tungstate followed by 1 ml. H_2SO_4 (50% (v/v)); this reduced the blank readings, which were very high for cereal products, by about 25%; (*b*) the use of 'Superfiltrol' (at pH 3) instead of Lloyd's reagent for adsorption; (*c*) the reading of the colour developed 2 min. after mixing, when the intensity was a maximum, in the Pulfrich photometer with filter S 57.

Table 6. *Comparison of values obtained for identical samples by the chemical, indophenol, method and microbiologically*

	Pyridoxin (μg./ml.)	
	Indophenol method	Yeast method
Beer, draught bitter	0·34	0·60
Beer, draught mild A	0·37	0·51
Beer, draught mild B	0·44	0·48
Beer, draught mild C	0·32	0·51
Stout	0·29	0·87
	(μg./g.)	
Brewery mixed grist (malted barley)	5·0	5·5

The method was originally developed to respond only to pyridoxin but was later (Melnick *et al.* 1945) shown to respond to a lesser extent to pyridoxal and pyridoxamine. Consequently the results obtained by this method have only a rough significance and the majority is not reported here. However, a few are shown in Table 6 with values obtained with the

same samples by the yeast method. As expected, the chemical values are lower and may give a rough indication of the proportion of the total vitamin B_6 present as pyridoxin.

SUMMARY

1. The microbiological method for the determination of vitamin B_6 with *Saccharomyces carlsbergensis* (Atkin *et al.* 1943) was satisfactorily applied, with slight modifications, to a number of foodstuffs.

2. Two chemical methods were also used and criticisms of them are presented.

3. Data obtained upon the effectiveness of different methods of extraction of vitamin B_6 from natural materials can be explained without assuming the existence of unknown compounds possessing vitamin activity.

4. Indications were obtained that the particular strain of *S. carlsbergensis* used might also be used for the estimation of biotin and inositol.

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Intercellular Hormones

6. RELEASE AND SYNTHESIS OF FACTORS OF THE VITAMIN B COMPLEX BY DAMAGED LIVING CELLS

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When cells are damaged under conditions which leave them grossly intact, proliferation-promoting factors ('wound hormones') are released into the intercellular suspension medium (see Cook & Cronin, 1941; Loofbourow, 1942*b* for earlier references). The mechanism of the effect appears to involve (*a*) increase in cell-membrane permeability, (*b*) diffusion of non-protein substances through the more permeable membranes, and (*c*) partial replacement within the injured cells of some of the substances lost through diffusion (Loofbourow, 1942*b*).

It has been suggested that the substances responsible for the proliferation-promoting activity might be amino-acids or vitamin B complex factors (Davidson, 1940), purines or pyrimidines (Loofbourow, Cook & Stimson, 1938) or combinations of nucleotides with vitamin B complex factors (Loofbourow, 1942*c*). Duplication of the proliferation-promoting effect of damaged-cell products by combinations of B complex factors, amino-acids, and adenine nucleotides has afforded presumptive evidence that all of these substances are involved in the effect (Loofbourow, 1942*b*).

To determine the role of any substance in the 'wound hormone' phenomenon it is desirable to establish the presence and relative concentration of the substance in products from damaged cells, and then to demonstrate its proliferation-promoting activity under like conditions of concentration and combination with other substances. The present paper presents quantitative assays of damaged-cell and undamaged-cell preparations with regard to factors of the vitamin B complex, together with growth tests employing B complex factors in comparable concentrations. Subsequent communications will deal with the role of amino-acids, nucleotides and nucleosides in the 'wound hormone' phenomenon.

METHODS

Preparation of products from damaged and undamaged cells. After thriee washing, by emulsification in volumes of 400 ml. of water and centrifugation, 400 g. wet weight (about 112 g. dry weight) of Fleischmann starch-free baker's yeast was suspended in distilled water to make a total volume of 4 l. Equal 2 l. amounts were placed in Pyrex glass containers. The two suspensions were stirred constantly by motor-

driven glass paddles. One suspension was irradiated for 8 hr. with lethal ultra-violet radiation from a Hanovia air-cooled d.c. quartz mercury arc operated at 65 V., 5·2 amp. and placed 24·5 cm. from the surface of the suspension. The non-irradiated suspension served as an undamaged-cell control. The irradiated suspension was maintained at approximately the same temperature as the non-irradiated suspension (22–23°) by means of a glass cooling coil. The depth of the suspensions in the Pyrex glass vessels was 6·3 cm. The lethal ultra-violet energy (wave-length shorter than 2950 A.) absorbed by the irradiated suspension during the 8 hr. irradiation period was approximately $4{\cdot}2 \times 10^8$ ergs/ml. of suspension. The cell volume before irradiation was 12·5% of the total volume of the suspension; after irradiation, it was 12% for the non-irradiated and 9% for the irradiated suspension. Details of the energy and cell-volume measurements are given in an earlier communication (Loofbourow, 1942*a*).

Following irradiation, both suspensions were centrifuged. The supernatant fluids were decanted, filtered through sterile Seitz G.P. pads, and autoclaved (15 min. at a pressure of 15 lb./sq.in.), the yield of intercellular fluids being 1842 ml. from the irradiated cells and 1765 ml. from the non-irradiated cells.

After centrifugation, the cell residues were washed thrice in 200 ml. volumes of water. The wash water from each residue was pooled, made up to 700 ml. with distilled water, and autoclaved.

To each washed sludge residue, an equal volume of distilled water and 5 ml. of toluene were added. To permit autolysis of the cell residues, the washed sludge residues were stored in the dark for 8 days at room temperature in loosely corked flasks, during which period they were shaken frequently. The acidity of autolysates was then adjusted to pH 2·7–2·8 with HCl, steamed for 30 min. at 100°, to afford further extraction of the cellular residue for B factors, and centrifuged. The supernatants were decanted, filtered through Seitz G.P. pads, and autoclaved.

Assays for B complex factors were carried out on the intercellular suspension fluids, the sludge-residue wash waters, and the autolyzed-cell extracts, in the case of both undamaged (control) cells and damaged (irradiated) cells.

Vitamin assay methods. Aneurin was assayed by the thiochrome method (Hennessy, 1941; Hennessy & Cerecedo, 1939). Nicotinic acid was assayed colorimetrically (Melnick & Field, 1940*a*, *b*; Melnick, 1941). A fluoriphotometric method was employed for riboflavin, following the technique of Hodson & Norris (1939) as modified in the laboratories of Prof. Robert S. Harris at the Massachusetts Institute of Technology. Biotin was assayed by the microbiological method of Williams (1941); 10 ml. cultures of yeast (*S. cerevisiae*, F.B. strain) were used. Pantothenic acid was assayed by Williams's (1941) yeast-growth method (using *S. cerevisiae*, G.M. strain) with the following modifications: (1) the cultures were grown in rotor tubes (Loofbourow, Webb, Loofbourow & Abramowitz, 1942), (2) an innoculum of 0·001 mg./ml. was used instead of 0·004 as used by Williams, (3) the medium supplements included a folic acid preparation, obtained from R. R. Williams, and casein in addition to the supplements employed by Williams, and (4) the pH was adjusted to 4·7. Folic acid was assayed by the microbiological growth method of Landy & Dicken (1941), using *L. casei* as the organism and the previously mentioned preparation of folic acid as the reference standard. The reference standard was an impure sample; the percentage of folic acid it was assumed to contain was based on data supplied by R. R. Williams. Both the Landy & Dicken (1941) *L. casei* growth method and the Williams (1941) yeast-growth method, with *S. cerevisiae* G.M. strain, were used for pyridoxin; the results reported here were obtained by Williams's method. Inositol was assayed by Williams's (1941) yeast-growth method, with *S. cerevisiae* G.M. strain.

The approximate errors of the assays (Tables 1 and 2) were determined by repeated assays of the same preparations in these and other experiments.

Determination of proliferation-promoting effects. The proliferation-promoting effects of the various preparations were determined by yeast-growth assays, with *S. cerevisiae*, F.B. strain, grown in rotor tubes (Loofbourow *et al.* 1942).

RESULTS

Vitamin assays. The results of the assays for vitamin B complex factors are shown in Table 1. In the case both of the intercellular fluids and of the sludge washings, the damaged cells gave greater yields of all factors assayed than did the undamaged cells. In the case of autolyzed sludge extracts, however, only biotin showed a greater yield from the damaged than from the undamaged cell sludge residue; the folic acid yield was the same, within experimental error, from both residues, while all of the remaining factors showed less yield from the damaged-cell sludge residue than from the undamaged-cell residue. The total yields in the intercellular fluids, wash waters, and sludge residue autolysates were significantly greater for damaged-cell preparations than for undamaged-cell preparations in the case of biotin, folic acid, and pantothenic acid. The total yields of inositol and nicotinic acid were nearly the same for damaged-cell and undamaged-cell products. In the case of pyridoxin, riboflavin, and aneurin, however, the total yields were significantly less from damaged than from undamaged cells. These lesser total yields were accounted for entirely by the lesser amounts in the sludge-residue autolysates.

Proliferation-promoting effects. It was reported previously (Loofbourow, 1942*b*) that the total proliferation-promoting potency of intercellular fluids and residue autolysates from slowly damaged cells is greater than that from undamaged cells. Such results suggest a replacement within the living, injured cells of proliferation-promoting factors lost to the intercellular fluids. However, in the previously reported experiments, the proliferation-promoting potencies of the intercellular fluids and of the residue autolysates were assayed separately and the numerical results were added. More conclusive data should result if the intercellular fluids and residue autolysates were combined and assayed together. Accordingly, in the present experiments, like portions of the intercellular fluids, wash waters, and residue autolysates were combined and assayed in the case

Table 1. *Assay of products from damaged and undamaged cells for B complex factors*

Factor	Source	Yield of factors (μg./g. of moist yeast in original suspension) Intercellular fluid	Sludge washings	Sludge autolysate extract	Total	Approximate error of assay (%)
Biotin	Irrad. susp.	0·120	0·0415	0·695	0·857	± 3
	NI* susp.	0·0095	0·0035	0·508	0·52	
	Ratio I*/NI	12·5	11·9	1·4	1·6	
Folic acid	Irrad. susp.	0·19	0·069	0·27	0·53	±10
	NI susp.	0·0025	0·005	0·27	0·28	
	Ratio I/NI	76·0	13·8	1·0	1·9	
Inositol	Irrad. susp.	124·0	105·0	625·0	854·0	± 5
	NI susp.	53·0	18·5	860·0	931·5	
	Ratio I/NI	2·36	5·7	0·76	0·9	
Nicotinic acid	Irrad. susp.	53·5	17·0	87·0	157·5	± 5
	NI susp.	6·18	2·1	132·0	140·3	
	Ratio I/NI	8·7	8·1	0·7	1·1	
Pantothenic acid	Irrad. susp.	36·0	9·9	13·4	59·3	± 5
	NI susp.	0·393	0·079	30·7	31·2	
	Ratio I/NI	91·5	125·0	0·44	1·9	
Pyridoxin	Irrad. susp.	3·3	0·81	23·0	27·1	± 7
	NI susp.	0·96	0·39	64·0	65·4	
	Ratio I/NI	3·4	2·1	0·36	0·41	
Riboflavin	Irrad. susp.	1·13	0·024	4·33	5·48	± 4
	NI susp.	0·068	0·012	9·15	9·23	
	Ratio I/NI	16·7	2·0	0·47	0·61	
Aneurin	Irrad. susp.	0·355	0·158	1·35	1·86	± 2
	NI susp.	0·079	0·035	3·55	3·66	
	Ratio I/NI	4·5	4·6	0·38	0·57	

* NI = non-irradiated suspension of yeast. I = irradiated suspension of yeast.

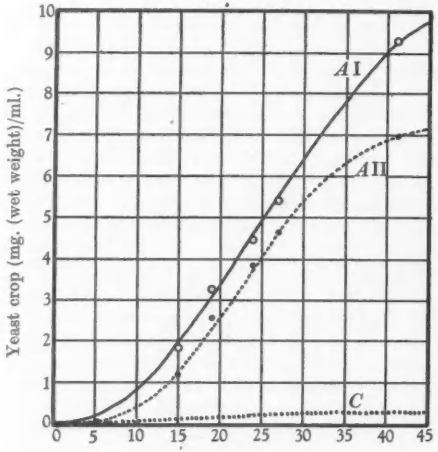

Fig. 1.

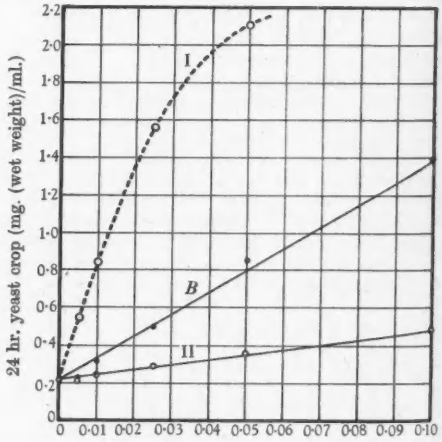

Fig. 2.

Fig. 1. Comparative yeast-growth curves obtained with combined samples of supernatant fluids, wash waters, and autolysates of cell residues from damaged cells (*A* I) and undamaged cells (*A* II). Each ml. of culture contained 0·2 ml. of the sample assayed. Growth in control cultures (Reader's medium only) is shown by curve *C*.

Fig. 2. Proliferation-promoting effect versus concentration for intercellular fluids from damaged cells (I) and from undamaged cells (II) in comparison with a solution of B complex factors (*B*) containing biotin, folic acid, inositol, nicotinic acid, pantothenic acid, pyrodoxin, riboflavin and aneurin in concentrations equivalent to those in (I).

both of products from damaged and from undamaged cells. The comparative proliferation-promoting effect of the combined samples is shown in Fig. 1. As in earlier experiments (Loofbourow, 1942*b*), the combined proliferation-promoting effect of products from damaged cells was greater than that of products from undamaged cells.

The 24 hr. growth obtained with various added quantities of intercellular fluids from damaged and undamaged cells is shown in Fig. 2, where also is shown, for comparison, the growth resulting from the addition of a solution containing B complex factors in concentrations corresponding to those found in the intercellular fluids from damaged cells (Table 2). The solution of vitamin B factors stimulated growth, but to an extent less than that of the intercellular fluids from damaged cells.

Table 2. *Content of B complex factors in intercellular fluids from damaged and undamaged cells*

	μg. of B factor/ml. of suspension fluid	
Factor	From undamaged cells	From damaged cells
Biotin	0·00108 ± 0·00003	0·013 ± 0·004
Folic acid	0·00028 ± 0·00003	0·020 ± 0·0002
Inositol	6·0 ± 0·3	13·5 ± 0·7
Nicotinic acid	0·70 ± 0·04	5·8 ± 0·3
Pantothenic acid	0·045 ± 0·002	3·9 ± 0·2
Pyridoxin	0·109 ± 0·008	0·35 ± 0·02
Riboflavin	0·0077 ± 0·00031	0·123 ± 0·005
Aneurin	0·009 ± 0·00018	0·039 ± 0·00077

The intercellular fluid volume obtained from the 2000 ml. of suspension of irradiated cells was 1842 ml. That from the 2000 ml. suspension of control cells was 1765 ml.

The relative growth-stimulating potencies of the intercellular fluids and of the solution containing B complex factors in concentrations corresponding to those found with intercellular fluids from damaged cells are shown in Table 3 in terms of PP units.* Approximately 18% of the potency of the intercellular fluids from damaged cells was obtained with the solution of B factors present in such fluids.

Table 3. *Proliferation-promoting potencies of intercellular fluids and of B factor equivalent of damaged-cell fluid*

One proliferation-promoting (PP) unit represents a 24 hr. yeast crop twice that of the control in 1 ml. of culture.

Preparation	ml. of prep./PP unit	PP units/ml.	Relative potency
Intercellular fluids from undamaged cells	0·083	12·2	1·0
Intercellular fluids from damaged cells	0·0032	312·8	25·9
Solution containing B complex factors in concentrations corresponding to those found in the intercellular fluids from damaged cells	0·018	55·6	4·5

DISCUSSION

The Seitz filter pads were examined for possible retention of fluorescing materials; no such retention was evident. Assays for riboflavin, aneurin and nicotinic acid at various stages of the procedure indicated that loss or destruction of these factors as a result of autoclaving and Seitz filtration was negligible. In the case of the other B factors, the extent of loss or destruction as a result of such treatment is not known, nor are data available as to the possible loss or destruction of B factors in the sludge autolysates as a result of standing at room temperature. However, the preparations from damaged cells were treated precisely the same as those from undamaged cells with regard to filtration, autoclaving, and temperature during autolysis. It would seem, therefore, that destruction or loss as a result of such treatments, if proportionate to the amounts of the substances present, would not influence the relative quantities of such substances in the final preparations from damaged cells as compared with those from undamaged cells.

With respect to irradiation, the conditions were not the same in the control and damaged-cell preparations. It is perhaps significant, therefore, that the instances in which appreciably lower total yields were obtained from damaged than from undamaged cells were those in which the factors concerned are known to be photochemically unstable, i.e. riboflavin, aneurin and pyridoxin.

Irradiation was carried out at about pH 5·5; hence conversion of riboflavin to lumichrome was to be expected. We attempted to assay lumichrome as well as riboflavin in the damaged-cell preparations by a fluorimetric method based on the difference in the fluorescent spectrum of lumichrome and riboflavin (Karrer, Salomon, Schöpp, Schlittler & Fritsche, 1934). This method was unsatisfactory, in part because lumichrome itself appears to be photochemically degraded into non-fluorescent products.

* The potencies of preparations assayed in previous investigations have been expressed in terms of 'growth units', one growth unit representing a 24 hr. yeast crop in 1 ml. of culture approximately 1000% greater than the control crop in a like volume of culture (i.e. a test-culture growth approximately 11 × the control-culture growth). It is preferable to adopt a smaller biological effect as the standard in order to avoid the necessity of extrapolations from the data obtained with undamaged-cell products and other low-potency materials. The proliferation-promoting unit (PP unit) employed in the present experiments represents a 24 hr. yeast crop 100% greater than that of the control in 1 ml. of culture. This unit corresponds to the 'relative growth unit' used by Cook & Cronin (1941).

The method did indicate, however, that the total amount of riboflavin and photochemical destruction products thereof in the damaged-cell preparations may have equalled or exceeded the total riboflavin content of the undamaged-cell preparations.

In the case of aneurin, computations were made of the photochemical destruction to be expected on the basis of a quantum efficiency for inactivation of the pyrimidine component of 0·0184 at $\lambda = 2537$ A. (Uber & Verbrugge, 1940*a*). Uber used a biological test for inactivation. His data indicate, however, that the initial photochemical change was a splitting off of the amino group in the 6-position. This would be expected to block the formation of thiochrome, and hence should lead to 'inactivation' in so far as the thiochrome assay, used in our experiments, is concerned. Uber found the thiazole component to be more sensitive photochemically (quantum efficiency for biological inactivation 0·347 at $\lambda = 2537$ A.), but the photochemical changes to which he attributed inactivation in this instance were not such as would be expected to interfere with thiochrome formation (Uber & Verbrugge, 1940*b*). From the energies in the 2500–2650 A. region employed in irradiation, the estimated absorption by interfering substances in the cells and suspension fluids, the above quantum efficiency for pyrimidine inactivation, and the average of the concentration of aneurin in damaged- and undamaged-cell preparations, it was computed that about 2·6 μg. of aneurin/g. of moist yeast may have been degraded as a result of photochemical action. The total yield for the damaged-cell preparations was 1·86 μg./g. of moist yeast (Table 1). This, together with 2·6 μg. assumed to have been photochemically degraded, leads to an hypothetical total of 4·46 μg. of aneurin/g. of moist yeast in the damaged-cell preparations as compared with 3·66 for the undamaged-cell preparations. Hence it seems possible that photochemical destruction may have accounted for the lesser total yield of aneurin from damaged as compared with undamaged cells (Table 1).

The results of these experiments are of primary interest in relation to the following three questions: (1) What factors may account for the increased proliferation-promoting effect of products from damaged cells as compared with those from undamaged cells? (2) Is there evidence for resynthesis of any of these factors within the living, damaged cells accompanying loss of these factors to the intercellular fluids? (3) Do the factors assayed in these experiments account entirely for the increased proliferation-promoting effect of products from damaged cells as compared with those from undamaged cells?

With regard to the first question, the data of Table 1 indicate that all of the factors determined may contribute to the increased proliferation-promoting effects of damaged-cell intercellular fluids (I) as compared with undamaged-cell intercellular fluids (II), since the quantities of the various factors were greater in all instances in I than in II. The assays establish only the possibility that each of these factors is important in the effect; the greater yields in I than in II are a necessary but not a sufficient condition for the contribution of the various factors to the proliferation-promoting effect. In the case of the combined samples of intercellular fluids, wash waters, and residue autolysates from damaged cells (*A* I) and from undamaged cells (*A* II), biotin, pantothenic acid, and folic acid were apparently the only ones of the factors assayed which could have contributed materially to the greater proliferation-promoting potency of *A* I, since only these were present in *A* I in significantly greater quantity than in *A* II, as indicated by the total yields in Table 1 (last column).

With regard to the second question, that of resynthesis, the data of Fig. 1 on growth assays of the combined samples *A* I and *A* II support the hypothesis that proliferation-promoting factors of some kind are produced, as well as released, during the course of cell damage. The assays in Table 1 show quantitatively a greater total yield of biotin, pantothenic acid, and folic acid from damaged cells and their suspension fluids than from undamaged cells and their suspension fluids, indicating elaboration of these substances by the damaged cells. Rogosa (1944) has found that, for normal yeast of the strain employed by us, both biotin and pantothenic acid (or its component β-alanine) are critical exogenous growth factors, indicating that the normal cells are incapable of elaborating such substances (at least in sufficient quantity to support optimum growth).

In considering the possibility of resynthesis of the various factors in the damaged cells, it should be borne in mind that the irradiated cells were not killed quickly but were damaged slowly. No appreciable cytolysis took place. The majority of the damaged cells were alive and metabolically active during most of the 8 hr. irradiation period. During this time, B factors and other proliferation-promoting substances were being released from the cells into the intercellular fluids, and it seems not unreasonable that replacement within the cells might have accompanied depletion resulting from diffusion into the suspending medium.

With regard to the third question, it appears that the factors assayed in the present experiments do not account entirely for the proliferation-promoting effects observed, as shown by the data of Fig. 2 and Table 3. This substantiates similar conclusions obtained by other methods (Cook & Cronin, 1941, 1942; Loofbourow, 1942*b*, *c*).

SUMMARY

1. Assays of the content of factors of the vitamin B complex of suspension fluids from ultra-violet damaged cells (I) and from undamaged cells (II)

showed greater amounts of biotin, folic acid, inositol, nicotinic acid, pantothenic acid, pyridoxin, riboflavin and aneurin in the former. The ratio of such factors in I as compared to II varied from a maximum of 91·5× for pantothenic acid to a minimum of 2·3× for inositol.

2. A solution of factors of the B complex corresponding to the quantities found in I stimulated the growth of yeast more than did II but not as much as did I, the relative proliferation-promoting potencies as compared with II being 25·9 for I and 4·5 for the B factor solution. It is concluded that factors of the vitamin B complex account for a part, but not all, of the proliferation-promoting effect in yeast-growth assays of products from damaged yeast cells.

3. Combined equal samples of the intercellular fluids, cell-residue wash waters, and residue autolysates from damaged cells (*A* I) and from undamaged cells (*A* II) were assayed for proliferation-promoting effect on yeast. Greater growth stimulation was obtained with *A* I than with *A* II. This confirms previously reported evidence that certain proliferation-promoting factors are replaced within the damaged cells as they are lost to the intercellular fluids by diffusion from the cells throughout the course of cell damage.

4. The total yield of factors of the B complex from intercellular fluids, residue wash waters, and residue autolysates was considerably greater from damaged than from undamaged cells in the case of biotin (1·6×), folic acid (1·9×) and pantothenic acid (1·9×) indicating that replacement within the living injured cells accompanies loss of these factors to the intercellular fluids. It is suggested that the instances in which lesser yields were obtained may possibly be explained by photochemical destruction in the irradiated (damaged-cell) preparations.

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Intercellular Hormones

7. RELEASE OF AMINO-ACIDS BY DAMAGED LIVING YEAST CELLS

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It has been demonstrated that factors of the vitamin B complex account in part for the proliferation-promoting activity ('wound hormone' effect) of products from yeast cells slowly damaged by sublethal ultra-violet radiation, and that the living, damaged cells appear to elaborate considerable quantities of certain of these factors (notably biotin and pantothenic acid) during the course of injury (Webb & Loofbourow, 1947).

Davidson (1940) found the amino-N content of suspension fluids from yeast cells damaged by ultra-violet radiation to be approximately 7 times that of suspension fluids from non-irradiated cells. This suggested that amino-acids may be involved in the

greater proliferation-promoting effect of the former, since it is well known that amino-acids are accessory growth factors for yeast (Mitchell & Williams, 1940). Many workers have concluded that polypeptides or amino-acids are involved in the increased proliferation of cells following tissue damage; the literature on this subject has been reviewed recently by Davidson (1943). Cook & Cronin (1941) found that addition to the culture media of various amino-acids suppressed to some extent the proliferation-promoting effect of products from ultra-violet irradiated yeast cells. In view of such results, it seemed of interest to investigate further the role of amino-acids in the proliferation-promoting effect of intercellular fluids from suspensions of cells subjected to controlled ultra-violet injury.

METHODS

The cellular products employed in these experiments were released by undamaged (control) and damaged (ultra-violet injured) yeast cells (*S. cerevisiae*, F.B. strain) into distilled water suspension media. The preparation methods are described in detail elsewhere (Loofbourow, 1942*a*; Webb & Loofbourow, 1947).

The total N content of the intercellular fluids was determined by the micro-Kjeldahl procedure, non-protein-N by the same procedure after deproteinization with sodium tungstate, and amino-N by formol titration and Folin's colorimetric method.

Analyses for specific amino-acids were made as follows: arginine was determined by a modified Sakaguchi reaction (Jorpes & Thoren, 1932), tyrosine and tryptophane by the Lugg method as modified by Brand & Kossel (1939), threonine and alanine by the method of Block & Bolling (1939*a*, *b*; Block, Bolling & Webb, 1940), and histidine by the diazo reaction (Koessler & Hanke, 1919) using Corning no. 502 filter for comparison with the colour developed in histidine reference solutions.

The proliferation-promoting effects of the suspension fluids from irradiated (I) and control cells (II), and of amino-acids (III), were determined by growth tests in Reader's (1927) medium with yeast (*S. cerevisiae*, F.B. strain) cultured in rotor tubes according to previously described techniques (Loofbourow, Webb, Loofbourow & Abramowitz, 1942).

RESULTS AND CONCLUSIONS

Nitrogen determinations were made upon four preparations each from damaged and undamaged cells, with results similar to those shown for preparation 627 in Table 1. Davidson's data (1940), recomputed to units of mg./ml., are included in Table 1 for comparison. Davidson's values are in all instances higher than ours. In particular, he found appreciable quantities of protein nitrogen in his preparations, whereas ours contained negligible amounts as indicated both by lack of precipitate with phosphotungstic acid, trichloroacetic acid or sodium tungstate and by the fact that our non-protein-N values were close to those for the total N. His conditions of preparation differed from ours, however, in that he employed: (i) twice the yeast concentration used in our experiments (as a result of which higher total solid and N contents would be expected in his preparations), (ii) a different ultra-violet source and time of irradiation, and (iii) 0·9 % NaCl as the suspension medium instead of distilled water.

Table 1. *Nitrogen determinations on suspension fluids from control and irradiated cells*

	Davidson's preparation			Preparation 627		
	I, Irr.* (mg./ml.)	II, NI* (mg./ml.)	Ratio I : II	I, Irr.* (mg./ml.)	II, NI* (mg./ml.)	Ratio I : II
1. Solid content	11·64	2·80	4·1	4·12	0·192	21·5
2. Total N	1·049	0·126	8·3	0·273	0·021	13·0
3. N.P.N.	0·962	0·105	9·2	0·272	†	—
4. Protein-N (by difference)	0·087	0·021	4·1	0·001	†	—
5. Amino-N	0·633	0·090	7·0	0·233	0·0167	13·9

* Irr. refers to cell-free suspension fluids from cells damaged by irradiation; NI to cell-free suspension fluids from non-irradiated cells.

† N.P.N. (non-protein nitrogen) was the same as total N in this material within the limits of experimental error.

Using the factor 6·25 to convert from amino-N to weight of amino-acids present, the indicated amino-acid content of preparation 627 I is 1·46 mg./ml. On this basis, assays for certain individual amino-acids other than alanine indicated their presence in this preparation in the following proportions relative to the total amino-acid content: arginine 1·7 %, histidine 2·3 %, threonine 4·9 %, tryptophan 0·7 %, and tyrosine 1·7 %. These values agree within experimental error with the distribution of amino-acids in yeast protein (Block & Bolling, 1940). In so far as these particular amino-acids are concerned, there was, therefore, no evidence for the release of amino-acids by the irradiated cells through any mechanism other than that of protein breakdown. Alanine, on the other hand, appeared to be present in I in considerably higher proportions than in yeast protein,

but the data were so variable as to be considered unreliable.

In the growth tests, various combinations of amino-acids in total concentrations varying from approximately 0·1–40 times the amino-acid content of I were tested in Reader's medium. The concentration of yeast in the cultures was determined turbidimetrically at various times; in a typical test, readings were made at 4, 9, 20, 24, 28, 33, 44, 50, 67 and 75 hr. Significant increases in the yeast crops, as compared with controls, were obtained only when the amino-acid concentration in the cultures was 30 or more times as great as that resulting from additions of I in amounts just sufficient to cause significant crop increases. These results confirm earlier conclusions (Cook & Cronin, 1941; Loofbourow, 1942*b*) that amino-acids do not account entirely for the proliferation-promoting activity of damaged-cell products under the conditions of our experiments.

The determination of the precise contribution of the amino-acid fraction to the 'wound-hormone' effect is complicated by the well-known fact that the increase in yeast crop obtained on addition of amino-acids varies with the presence or absence of other substances in the medium. This is illustrated by the results in Table 2. In the series of experiments of which this is a typical example, the amino-acid mixture (III) consisted of equal parts by weight of arginine, asparagine, aspartic acid, glutamic acid and leucine. Only these amino-acids and β-alanine appear from our experiments to be appreciably effective in increasing the growth of our strain of yeast, confirming previous results of Cook & Cronin (1941). β-Alanine was omitted from the amino-acid mixture employed because it acts as a critical exogenous growth factor for *S. cerevisiae*, F.B. strain, in lieu of pantothenic acid in media unsupplemented with B factors (Rogosa, 1944).

The basal media employed were (1) Reader's unsupplemented, (2) Reader's supplemented with the following (concentrations in μg./ml. of culture medium): aneurin hydrochloride, 0·25; riboflavin, 0·5; pyridoxin, 0·5; calcium pantothenate, 0·125; biotin (free base), 0·000125; choline, 0·75; and nicotinamide, 2·5.

Addition of 0·313 mg. of III/ml. of final culture to yeast grown in Reader's medium resulted in increases in the 28 and 44 hr. yeast crops of 0·048 and 0·040 mg./ml. (wet weight of yeast) respectively (Table 2). When, however, the same amount of III was added to cultures grown in Reader's medium supplemented with B factors, the increases in 28 and 44 hr. crops were respectively 1·57 and 1·72 mg./ml. Thus, amino-acids probably play a more significant quantitative role in the 'wound hormone' effect under the conditions of our investigations than is indicated by experiments in which amino-acids alone are tested for growth-stimulating effect in Reader's medium.

Table 2 also shows that the addition to yeast cultures, grown in Reader's medium supplemented with B factors, of 0·159 mg. of I‡/ml. of final culture *plus* 0·313 mg. of III resulted in greater increased 28 and 44 hr. crops than addition of 0·313 mg. of III alone. The increased 28 and 44 hr. crops were respectively 2·87 and 3·07 in the former case as against 1·57 and 1·62 in the latter. This confirms earlier evidence (Cook & Cronin, 1941; Loofbourow, 1942*b*)

Table 2. *Increased growth resulting from addition of amino-acids and damaged-cell products to yeast cultures*

Basal medium	Added material (mg./ml. of culture)	Yeast crop (mg./ml. wet weight) 28 hr.	Yeast crop (mg./ml. wet weight) 44 hr.	Increased yeast crop resulting from added material 28 hr.	Increased yeast crop resulting from added material 44 hr.
(1) Reader's	(*a*) None	0·152	0·157	—	—
	(*b*) 0·313 mg. amino-acids (III)*	0·200	0·197	0·048	0·040
	(*c*) 0·159 mg. I†	4·03	4·43	3·88	4·27
(2) Reader's + B factors (see text)	(*a*) None	3·33	3·70	—	—
	(*b*) 0·313 mg. amino-acids (III)*	4·90	5·42	1·57	1·72
	(*c*) 0·313 mg. amino-acids (III)* + 0·159 mg. I†	6·20	6·77	2·87	3·07

* III refers to equal parts by weight of arginine, asparagine, aspartic acid, glutamic acid and leucine.
† I refers to cell-free suspension fluid from yeast cells damaged by ultra-violet radiation.

The basal media employed were (1) Reader's unsupplemented, (2) Reader's supplemented with the following (concentrations in μg./ml. of culture medium): aneurin hydrochloride, 0·25; riboflavin, 0·5; pyridoxin, 0·5; calcium pantothenate, 0·125; biotin (free base), 0·000125; choline, 0·75; and nicotinamide, 2·5.

Addition of 0·313 mg. of III/ml. of final culture to yeast grown in Reader's medium resulted in increases in the 28 and 44 hr. yeast crops of 0·048 and 0·040 mg./ml. (wet weight of yeast) respectively (Table 2). When, however, the same amount of III was added to cultures grown in Reader's medium supplemented with B factors, the increases in 28 and 44 hr. crops were respectively 1·57 and 1·72 mg./ml. Thus, amino-acids probably play a more significant quantitative role in the 'wound hormone' effect under the conditions of our investigations than is indicated by experiments in which amino-acids alone are tested for growth-stimulating effect in Reader's medium.

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SUMMARY

1. Cell-free suspension fluids from damaged (ultra-violet injured) cells (I) had appreciably higher content of amino-N than fluids from control suspensions (II).

2. Amino-acids (III) in concentrations equivalent to or greater than that in I stimulated the growth of

‡ I.e. by addition of a volume of I containing 0·159 mg. solids.

yeast in unsupplemented Reader's medium and in Reader's medium supplemented with B factors.

3. Addition of I to Reader's medium stimulated the growth of yeast more than addition of III in all concentrations employed. Addition of I and III to Reader's medium supplemented with B factors stimulated the growth of yeast more than addition of III alone.

4. It is concluded that amino-acids account for a part, but not all, of the proliferation-promoting effect of I.

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Intercellular Hormones

8. RELEASE OF NUCLEOTIDES AND NUCLEOSIDES BY DAMAGED LIVING CELLS

By J. R. LOOFBOURROW, S. OPPENHEIM-ERRERA,* DOROTHEA G. LOOFBOURROW and CONSTANCE A. YEATS

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The chemical mechanism whereby the rate of proliferation of normal cells is increased in the neighbourhood of damaged cells (sometimes referred to as the wound-hormone phenomenon) involves several parameters, including: (1) the nature of the cells involved, (2) the method, extent, and duration of cell damage, and (3) the nature of the intercellular *milieu*. In preceding communications, we have presented evidence that in the special case of yeast cells damaged by prolonged sublethal dosages of ultraviolet radiation in a distilled water suspension medium, members of the vitamin B complex, amino-acids and nucleotides are released in considerable quantities into the suspension medium without appreciable cytolysis (Loofbourow, 1942*a*, *b*, 1947; Webb & Loofbourow, 1947).

With regard to the vitamin B complex ('B factors'), it appears that the living, damaged yeast cells elaborate biotin, pantothenic acid and folic acid in appreciable quantities during the course of cellular damage (Webb & Loofbourow, 1947). The strain of yeast we have employed in these experiments (*S. cerevisiae*, F.B. strain) is, according to Rogosa (1944), incapable of synthesizing pantothenic acid in normal growing cultures in sufficient quantities to support growth; hence it appears that the synthetic processes in damaged living cells may differ materially from those in normal cells.

The increased content of 'B factors' and amino-acids in the suspension fluids from damaged cells (I) as compared with those from control cells (II) accounts in large measure, but not entirely, for the increased proliferation-promoting effect of the former (Cook & Cronin, 1941; Webb & Loofbourow, 1947; Loofbourow, 1947). When, however, adenine nucleotides are added to yeast cultures in combination with B factors and amino-acids in appropriate concentrations, the proliferation-promoting effect of I can be duplicated in its entirety (Loofbourow, 1942*b*). This fact, together with various spectroscopic and chemical evidence for the presence of

* Deceased.

nucleotides and nucleosides in I (Loofbourow, Cook & Stimson, 1938; Davidson, 1940) indicates that nucleotides or substances related thereto are involved in the proliferation-promoting effect of I.

The present investigations afford further evidence as to: (1) the nature of the nucleotides and nucleosides present in I, (2) the role of such substances in the proliferation-promoting activity of I, and (3) the possibility of synthesis of such substances within the living damaged cells during the course of injury.

METHODS

To prepare cell-free suspension fluids from damaged and undamaged cells, washed, starch-free, moist yeast (*S. cerevisiae*, F.B. strain) was suspended at 100 g. (wet weight)/l. of suspension in distilled water, and divided into 2 l. samples. For the preparations from damaged cells, the 2 l. samples were irradiated with full ultra-violet from a quartz mercury arc for 8 hr. periods. During these periods, the undamaged-cell samples were maintained under the same conditions of temperature, stirring, etc. Following irradiation, the cell-free suspension fluids were obtained from irradiated (I) and non-irradiated (II) cells by centrifugation and Seitz filtration. Further details of the preparation methods are given in earlier communications (Loofbourow, Webb, Loofbourow & Abramowitz, 1942; Loofbourow, 1942*b*).

In all, six preparations each of I and II were employed in the investigations reported here. Three of the preparations were autoclaved at a pressure of 15 lb./sq.in. for 20 min. at pH about 6 following Seitz filtration; three were not autoclaved. There was no indication that autoclaving caused appreciable degradation of the nucleotides present, but this question requires further investigation.

The above method of preparing suspension fluids from damaged yeast cells yields a sparkling-clear, straw-coloured solution, substantially free from protein. There is inappreciable cytolysis of the damaged cells, the substances which enter the intercellular fluids apparently doing so by diffusion through the intact cell membranes, made more permeable as a result of cellular damage. The damaged cells are alive during most of the irradiation period, as determined by the methylene blue staining reaction, and are capable of carrying on synthetic processes, as indicated by their tendency to form buds (Loofbourow, 1942*a*). The synthetic processes which take place during the course of irradiation under these conditions must, of course, be supported by substances already present in the cells, since the suspending medium is water.

Amino-N was determined by formol titration and Folin's colorimetric procedure. All other N determinations were made by the micro-Kjeldahl procedure: non-protein-N (N.P.N.) after precipitation of protein with sodium tungstate; nucleotide and nucleoside and free purine-N after separation of these fractions according to the method of Kerr & Blish (1932; Kerr, 1940).

Free and bound P were determined by the method of Fiske & Subbarow (1925).

The proliferation-promoting potencies of the various samples were assayed on yeast (*S. cerevisiae*, F.B. strain) grown in rotor tubes according to previously described procedures (Loofbourow *et al.* 1942). Two media were used: (1) Reader's (1927), unsupplemented, and (2) the same, supplemented with the following growth factors (concentrations in μg./ml. of medium): aneurin hydrochloride, 0·25; riboflavin, 0·5; pyridoxin, 0·5; calcium pantothenate, 0·125; biotin (free base), 0·000125; choline, 0·75; nicotinamide, 2·5; arginine, 625; asparagine, 625; aspartic acid 625; glutamic acid, 625; and leucine, 625.

Ultra-violet absorption spectra were determined with a Hilger quartz spectrograph and Spekker photometer.

RESULTS

The results of analyses for nucleotide-N and nucleoside- and free purine-N of the cell-free suspension fluids of preparation 627 are shown in Table 1. Analyses of other preparations gave similar results; e.g. the cell-free suspension fluids from irradiated

Table 1. *Analyses of cell-free suspension fluids from irradiated and non-irradiated cells*

	Davidson (1940)			Preparation 627		
	I, Irr.* (mg./ml.)	II, NI* (mg./ml.)	I/II	I, Irr.* (mg./ml.)	II, NI* (mg./ml.)	I/II
1. Solid content	11·64	2·80	4·1	4·12	0·192	21·5
2. Nucleotide-N	0·118	0·004	29·5	0·0155	0·00045	34·4
3. Nucleoside and free purine-N	0·094	0·014	6·7	0·0214	0·0040	5·4
4. Amino-N	0·633	0·090	7·0	0·233	0·0167	13·9
5. Totals 2, 3 and 4	0·845	0·108	—	0·270	0·0212	—
6. N.P.N.	0·962	0·105	9·2	0·272	0·021	12·9
7. Ultra-violet absorption in 0·1 N-HCl:						
(*a*) $\lambda_{max.}$	—	—	—	2570 A.	2650 A.	—
(*b*) $1/x \log_{10} I_0/I_x$	—	—	—	8·8	1·9	4·6

* Irr. refers to suspension fluids from irradiated cells. NI refers to suspension fluids from non-irradiated cells.

and non-irradiated cells in preparation 456 had nucleotide-N values of 0·0159 and 0·00047 mg./ml. respectively.

In comparing our results with those of Davidson (1940), which have been recomputed from his data to terms of mg./ml. and included in Table 1, it should be noted that his preparations were obtained from yeast suspensions twice as concentrated as ours, so that his values per ml. of suspension fluid would be expected to be larger than ours. Furthermore, his conditions of irradiation were different, and the strain of *S. cerevisiae* he employed may not have been identical with the one we used. Comparison of our 627 preparation with his on the basis of the ratios (I/II) of the analyses of suspension fluids from irradiated cells (I) to those of suspension fluids from non-irradiated cells (II) shows considerable similarity in so far as nucleotide and nucleoside-N are concerned. The nucleotide-N ratios (I/II) were particularly high, being 29·5 for Davidson's preparation, 34·4 for our preparation 627, and 39·5 for our preparation 456 (not shown in the table).

Davidson found greater quantities of nucleotide-N than of nucleoside and free purine-N in I. The reverse was true in all of our preparations. This may indicate that there was greater degradation of nucleotides to nucleosides and free purines under our conditions of irradiation and fractionation.

In endeavouring to isolate the nucleotide fraction, we were led by the following considerations to undertake the separation of adenosine triphosphate (ATP) from the preparations: (1) early investigations (Loofbourow *et al.* 1938; Cook, Loofbourow & Stimson, 1939) indicated that the ultra-violet absorption of I was attributable primarily to adenine and possibly to some extent to guanine; (2) Davidson (1940) isolated adenine as the picrate and sulphate from his preparations of I; small quantities of guanine were also found to be present; (3) in growth tests (Loofbourow, 1942*b*), the proliferation-promoting effect of I was duplicated with combinations of amino-acids, B factors, and ATP; neither muscle adenylic acid nor adenosine was found to be as effective as ATP; (4) the ratios of purine-N to organic P for preparation 225 I ranged from 0·8 to 0·9, suggesting the presence of nucleotides of high P content, such as ATP (N/P = 0·75) or adenosine diphosphate (N/P = 1·13). In view of these considerations, an endeavour was made to isolate the barium salt of ATP from I by the method of Kerr (1941), even though it appeared unlikely that ATP would be present in I in appreciable quantities following the usual 8 hr. irradiation period at room temperature.

In the ATP preparations, all manipulations following irradiation, including centrifugation and Seitz filtration to free the suspensions from cells, were carried out in a cold room at 4° or with the preparations cooled by an ice-bath. In two preparations, the yield of ATP was negligible. Control preparations from freshly killed rabbit muscle gave satisfactory yields of ATP in both instances. Ultra-violet absorption spectra indicated that almost the entire purine fraction remained in the supernatants of the yeast preparations following precipitation of ATP by barium acetate and ethanol.

In further treatment of supernatants obtained by the above procedure or slight modifications thereof, the nucleotide fraction was precipitated with mercuric acetate, resuspended, freed from mercury and precipitated repeatedly from water solution by addition of acetone.

Fig. 1 shows the similarity of the ultra-violet absorption spectrum of one such preparation (N_1) to a typical absorption spectrum of an adenine nucleotide (BaATP from rabbit muscle). Fig. 2 compares the absorption of N_1 with that of a later preparation by the same method (N_2). Variability in spectra to the extent indicated by this comparison was characteristic of various preparations made by this method; such variability is probably attributable to differences in the purity of the various preparations.

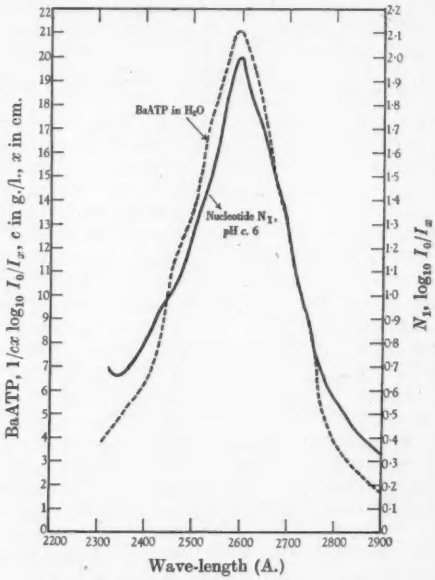

Fig. 1. Ultra-violet absorption spectrum of nucleotide (N_1) prepared by Hg-Ba-acetone precipitation from cell-free fluid from irradiated cells, compared with the absorption of BaATP prepared from rabbit muscle. The concentration of the nucleotide solution was adjusted to give a maximum density, $\log_{10} I_0/I_x$, of 2 to facilitate comparison of the shapes of the curves.

Fig. 2 also shows that changes in pH had little effect on the ultra-violet absorption of the acetone-precipitated nucleotide fraction, a characteristic which would be expected if the absorption were attributable to adenine (Holiday, 1930; Loofbourow & Stimson, 1940; Stimson & Reuter, 1945).

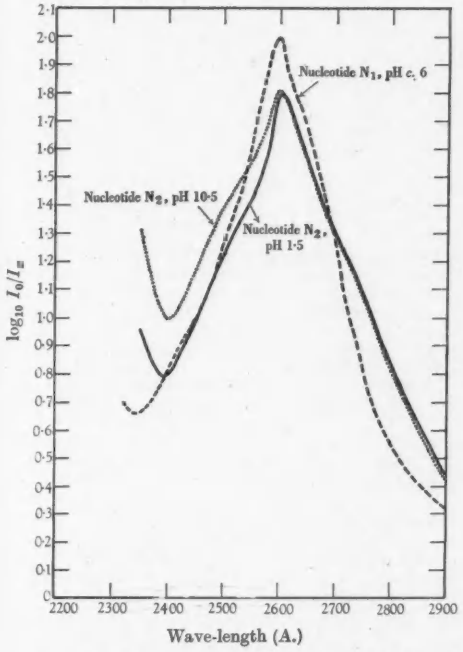

Fig. 2. Ultra-violet absorption spectra of nucleotides N_1 and N_2 prepared by Hg-Ba-acetone precipitation from cell-free fluids from irradiated cells. The concentration of N_1 was adjusted to give a maximum optical density, $\log_{10} I_0/I_x$, of 2. The two curves showing effect of pH on N_2 are for identical concentrations of N_2, this concentration being chosen so as to give a maximum optical density of about 1·8 to facilitate comparison of the shapes of the curves with that of N_1. In determining the spectra of N_2, acid or alkali was added to the comparison cell in the same concentration as employed in the solution undergoing measurement.

Two of the acetone-precipitated nucleotide preparations (N_1 and N_2) were tested for proliferation-promoting activity in Reader's medium, unsupplemented and supplemented with B factors and amino-acids. The results are summarized in Table 2. In all concentrations employed, the nucleotide fractions stimulated the growth of yeast, both in the unsupplemented and the supplemented Reader's media. Addition of the nucleotides to the supplemented medium in concentrations such that their absorption at 260 A. after dilution by the medium was equivalent to that of $3{\cdot}7 \times 10^{-6} - 1{\cdot}85 \times 10^{-5}$ mol. of adenine/l., resulted in yeast crops closely similar to those obtained by addition to unsupplemented Reader's medium of 0·05 ml. of preparation 627 I/ml. of culture (corresponding in ultra-violet absorption at 2600 A. to $3{\cdot}24 \times 10^{-5}$ mol. of adenine/l. of culture). This indicates that the nucleotides and related compounds in I account for the additional proliferation-promoting effect of I above that attributable to amino-acids and B factors.

Two further preparations of nucleotide were made following the procedure described above except that the nucleotide was precipitated by addition of acetone to a water solution containing the barium salt. One of these preparations was tested for growth activity with results similar to those shown for preparations N_1 and N_2 in Table 2. The spectra of both preparations were characteristic of adenine derivatives, as in Fig. 1. The ratios of purine-N to bound P for these two preparations were respectively 1·0 and 1·6.

The yields by the Hg-Ba-acetone precipitation method were of the order of 10–20 % of the theoretical purine content of the starting material as determined spectrographically. Because of the low yield, no further attempt was made to isolate the nucleotide in this manner. Instead, preliminary studies were made of the nucleoside and nucleotide fractions isolated for N determinations by uranium and copper salt precipitation according to the method of Kerr & Blish (1932; Kerr, 1940).

Adenine was estimated by the method of Hitchings (1933) after isolation as the picrate from the nucleotide fraction. Guanine was estimated by a quantitative colorimetric procedure based on the diazo reaction. The results indicated that at least 85 % of the purine content of the nucleotide fraction was adenine, the remainder probably being guanine. Satisfactory quantitative data were not obtained for the nucleoside fractions; adenine and guanine also appeared to be present therein, but the possibility of other purines or pyrimidines being present in substantial quantities was not excluded.

Positive tests for ribose were obtained with both the nucleotide and nucleoside materials. Tests for deoxyribose were negative.

The spectrum of the nucleotide fraction at acid pH (approximately 0·1 N-HCl) was similar to that of adenine and its nucleotide and nucleoside derivatives (Fig. 3). The spectrum of the nucleoside fraction at similar pH had a maximum at 2550 A. rather than at 2600–2610 A., the latter being characteristic of adenine.

The nucleotide and nucleoside fractions obtained by the Kerr & Blish procedure accounted for nearly all of the purine content of I as indicated by the N balance sheet (Table 1) and by comparison of the

Table 2. *Proliferation-promoting effect of nucleotide fractions prepared by Hg-Ba-acetone precipitation*

Fractions N_1 and N_2 are derived from the cell-free fluids from irradiated cells (see text).

Medium	Addition to medium per ml. of culture	Equivalent adenine in culture (mol./l.)‡	Yeast crop (mg./ml. wet weight)		Increased crop resulting from added material	
			24 hr.	34½ hr.	24 hr.	34½ hr.
Reader's	None	—	0·25	0·25	—	—
	0·1 ml. Nucleotide N_1	$3·7 \times 10^{-5}$	1·07	1·66	0·82	1·41
	0·05 ml. Nucleotide N_1	$1·85 \times 10^{-5}$	0·63	0·95	0·38	0·70
	0·02 ml. Nucleotide N_1	$7·4 \times 10^{-6}$	0·40	0·71	0·15	0·46
	0·01 ml. Nucleotide N_1	$3·7 \times 10^{-6}$	0·31	0·50	0·06	0·25
	0·05 ml. Nucleotide N_2	$1·85 \times 10^{-5}$	0·53	1·39	0·28	1·14
	0·05 ml. Prep. 627 I†	$3·24 \times 10^{-5}$	3·44	5·40	3·19	5·15
	0·05 ml. Prep. 627 NI†	$6·9 \times 10^{-6}$	0·62	0·71	0·37	0·46
Reader's + B factors + amino-acids*	None	—	3·02	4·35	—	—
	0·05 ml. Nucleotide N_1	$1·85 \times 10^{-5}$	3·70	6·30	0·68	1·95
	0·01 ml. Nucleotide N_1	$3·7 \times 10^{-6}$	3·25	5·40	0·23	1·05
	0·05 ml. Nucleotide N_2	$1·85 \times 10^{-5}$	3·21	6·14	0·19	1·79

* Reader's medium supplemented with the following growth factors (concentrations in μg./ml. of medium): aneurin hydrochloride, 0·25; riboflavin, 0·5; pyridoxin, 0·5; calcium pantothenate, 0·125; biotin (free base), 0·000125; choline, 0·75; nicotinamide, 2·5; arginine, 625; asparagine, 625; aspartic acid, 625; glutamic acid, 625; and leucine, 625.

† Irr. refers to cell-free suspension fluid from irradiated cells. NI refers to cell-free suspension fluid from non-irradiated cells.

‡ The solutions of N_1 and N_2 were made up at such concentrations as to have optical densities ($1/x \log_{10} I_0/I_x$) of 5·0 at $\lambda = 2600$ A., equivalent to adenine concentrations of $3·7 \times 10^{-4}$ mol./l. if all absorption at this wave-length were attributable to adenine. The optical densities at 2600 A. of preparations 627 I and 627 NI were respectively 8·8 and 1·9, equivalent to adenine concentrations of $6·48 \times 10^{-4}$ and $1·38 \times 10^{-4}$ mol./l. respectively, assuming all absorption at 2600 A. to be attributable to adenine.

Table 3. *Proliferation-promoting effect in Reader's medium of nucleotide and nucleoside fractions prepared as copper salts*

Addition to medium	Equivalent adenine in cultures (mol./l.)*	Yeast crop (mg./ml. wet weight)		Increased crop resulting from added material	
		24 hr.	39½ hr.	24 hr.	39½ hr.
None	—	0·28	0·40	—	—
Nucleotide fraction	$4·42 \times 10^{-5}$	0·43	0·66	0·15	0·26
	$2·21 \times 10^{-5}$	0·37	0·51	0·09	0·11
	$1·11 \times 10^{-5}$	0·37	0·48	0·09	0·08
	$5·55 \times 10^{-6}$	0·35	0·40	0·07	0·00
Nucleoside fraction	$1·18 \times 10^{-5}$	0·53	0·82	0·25	0·42
	$5·90 \times 10^{-6}$	0·42	0·51	0·14	0·11
	$2·95 \times 10^{-6}$	0·35	0·43	0·07	0·03
	$1·48 \times 10^{-6}$	0·35	0·42	0·07	0·02

* The nucleotide and nucleoside solutions were added to the cultures in such concentrations as to have ultra-violet absorption at 2600 A. corresponding to the absorption of adenine at the molar concentrations shown.

summation of the spectra of these fractions with the spectrum of I (Fig. 3). In plotting Fig. 3, the optical densities of the nucleotide and nucleoside solutions were divided by the number of ml. of starting material (I) per ml. of each solution, so as to obtain data representing the contribution of the absorption of each of these fractions to the absorption of the original material. From data as to the content of tyrosine, tryptophan, and absorbing B factors in I (Loofbourow, 1946; Webb & Loofbourow, 1946) it was computed that such substances might account for an optical density of about 0·6 at 2560 A. The net optical density of I at 2560 A. after subtracting the absorption of such materials is 8·25, as compared with 7·9 for the nucleotide-nucleoside summation curve.

Growth tests of the Cu-free nucleotide and nucleoside fractions in Reader's medium are summarized in Table 3. Both fractions had appreciable activity at the higher concentrations employed. The activity of the nucleoside was apparently somewhat greater than that of the nucleotide. Neither fraction appeared to be as active in Reader's medium as the Hg-Ba-acetone preparations at concentrations corresponding to equivalent ultra-violet absorption at 2600 A.

The total absorption at 2600 A. of the cell-free intercellular fluids and residue cells was determined in several preparations from irradiated and non-irradiated yeast after release of the contents of the cell residues by autolysis or by digestion with strong alkali. The absorption at 2600 A. was in all instances greater in the case of the total intracellular and extracellular materials from irradiated cells (I A) than in the case of like materials from non-irradiated cells (II A). The ratio of absorption (I A/II A) at 2600 A. in

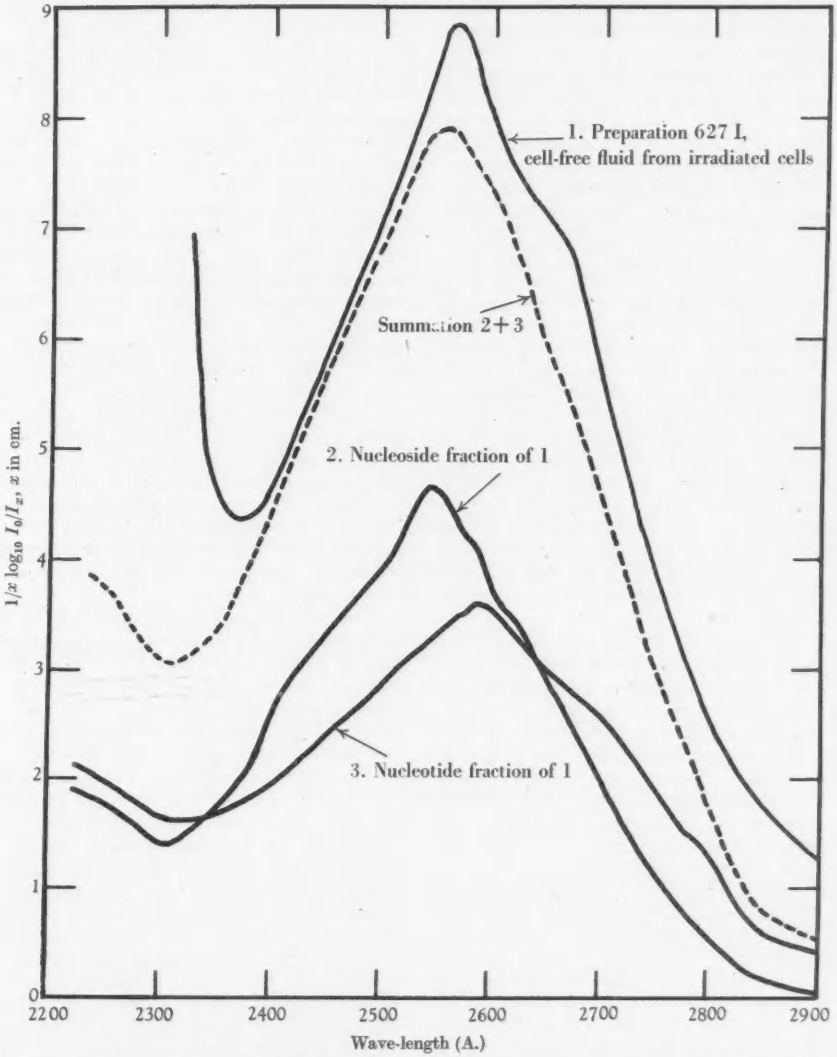

Fig. 3. Ultra-violet absorption spectrum of the cell-free fluid from irradiated cells (prep. 627 I) in comparison with the spectra of the nucleotide and nucleoside fractions thereof obtained by the Kerr & Blish (1932), Kerr (1940) procedure. The absorption coefficients, $1/x \log_{10} I_0/I_x$, of the two fractions are shown for concentrations in which each ml. of the nucleotide or nucleoside solution corresponds to 1 ml. of the cell-free preparation (627 I) from which it was prepared.

a typical preparation was 1·9. If the absorption at 2600 A. is assumed to be a measure of the purine-pyrimidine content of the preparations, these data indicate that such materials were synthesized by the living, damaged cells during the course of injury. The data further indicated that only about 10 % of the total absorption at 2600 A. in irradiated cell preparations is accounted for by the absorption of the cell-free suspension fluids; thus most of the increase in total absorption of irradiated as compared with non-irradiated preparations is attributable to substances which accumulate within the cells and are not released into the intercellular fluids.

DISCUSSION

The results reported here afford direct evidence of what has long been suggested by less direct experiments: nucleotides and nucleosides account for that part of the proliferation-promoting effect of cell-free fluids from suspensions of yeast cells slowly damaged by ultra-violet radiation which is not accounted for by amino-acids and B factors.

The exact nature of the nucleotides and nucleosides responsible for the effect remains to be established. The evidence indicates that adenine derivatives play a major role, that guanine derivatives and possibly other purine and pyrimidine derivatives may also be involved, and that the nucleotides and nucleosides are of the ribose rather than the deoxyribose type.

Recent investigations of Englert (1945) have shown that hydrolysis products of ribonucleic acid stimulate the growth of yeast and have led her to suggest that nucleosides released in this way (in particular adenosine and guanosine) account in part for the proliferation-promoting activity of damaged-cell preparations. From preliminary growth experiments other than those reported here, we are inclined to believe, however, that the most active components in the damaged-cell preparations are nucleotides rather than nucleosides.

The absorption spectra of the total extracellular and intracellular material indicate that nucleotide-like substances are synthesized, as well as released into the intercellular fluids, by cells slowly damaged by ultra-violet irradiation. This is consistent with the increase in ultra-violet absorption at 2600 A. which has been noted in the cytoplasm of cells irradiated and observed periodically on the stage of a quartz microscope (Loofbourow & Joyce, 1941) and with the increased yield of ribonucleic acid previously found for irradiated as compared with non-irradiated yeast (Loofbourow *et al.* 1941; Englert, 1942, 1945). Mitchell (1942) reported a similar increase in the content of ribonucleic-acid-like substances in the cytoplasm of living cells exposed to Roentgen radiation or gamma rays.

It is perhaps not surprising that the synthetic processes in living cells should undergo some degree of alteration, quantitatively or qualitatively, when the cells are subjected to various damaging agents under circumstances which do not lead to rapid cell death, thus leading to increased quantities of particular substances within the cell and (in the case of substances capable of diffusing through the cell membrane) in the intercellular *milieu*.

After a careful review of the wound-hormone problem, Davidson (1943) appears to favour the hypothesis that the only substances set free by damaged cells are breakdown products of the cellular protoplasm, and that there is no synthesis of liberated components within living, injured cells during the injury period. We are inclined to disagree with Davidson on this point since: (1) data with regard to B factors (Webb & Loofbourow, 1947) indicate rather clearly an increased production, as well as release, of some of these factors during the course of injury; (2) the increased extracellular and intracellular absorption at 2600 A. in the case of damaged as compared with undamaged cells is presumptive evidence for more rapid synthesis of purine-pyrimidine-like materials in the injured cells; (3) it is difficult to conceive of a mechanism whereby a dynamic chemical system, like that which occurs in living cells, should maintain a *status quo* despite the drastic changes in conditions imposed by exposure to lethal radiation.

When we first undertook a study of the 'wound hormone' problem, some years ago, we expected to find, as Davidson suggests, that dead-cell disintegration products accounted entirely for the phenomenon of increased growth of normal cells in the presence of products from damaged cells. The early observation that products from slowly injured cells had greater proliferation-promoting activity than products from quickly killed cells (Loofbourow, Dwyer & Lane, 1940) suggested, however, that in addition to disintegration of dead cells, changes in the metabolism and related synthetic processes of living, injured cells might be involved in the phenomenon. While the hypothesis that cell death and disintegration are the only factors involved in the 'wound hormone' phenomenon is an attractive one because of its simplicity, we believe, in view of our experiments, that such an hypothesis is an over-simplification in instances in which the damaged cells continue to live for an appreciable period of time.

SUMMARY

1. An investigation was made of the role of nucleotides and nucleosides in the increased proliferation-promoting activity of cell-free suspension fluids from cells slowly damaged by lethal radiation (I) as compared with similar fluids from undamaged cells (II).

The nucleotide-N and nucleoside- and free purine-N contents of I were respectively about 34 and 5 times those of II. Nucleotides isolated from I, when combined with amino-acids and B-factors found in I, duplicated the proliferation-promoting effect of I.

2. The nucleotide and nucleoside fractions were found to consist principally of adenine and guanine derivatives.

3. Spectroscopic evidence indicated a synthesis of materials absorbing at 2600 A. within the living, injured cells during the course of injury.

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The Quantitative Separation on the Micro-scale of Phenylarsenious Acid, Phenylarsonic Acid, Arsenious Acid and Arsenic Acid in Simple Solution and in Urine

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It has been generally assumed that aromatic arsonic acids undergo reduction *in vivo* to the corresponding arsenoxides before producing their parasiticidal or toxic actions. All evidence for this is, however, of an indirect nature (Voegtlin & Smith, 1920; Yorke & Murgatroyd, 1930; Murgatroyd, Russell & Yorke, 1934; Thuret, 1938). It has also been suggested that arsenoxides are oxidized before excretion in the urine (Chance, Crawford & Levvy, 1945). Evidence as to whether or not inorganic arsenic derivatives are formed from organic arsenicals in the body is scanty and inconclusive. Definite information regarding the metabolism of organic arsenicals has awaited the development of suitable microchemical methods.

To investigate the changes undergone in the body by phenylarsenious acid and phenylarsonic acid, a method was required whereby each constituent of a mixture of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid when present as their salts in biological material could be determined on the micro-scale. It was considered that such a method could be developed incorporating the procedure previously described (Crawford & Levvy, 1946) for the separation of the trivalent and pentavalent arsenic compounds, provided that extraction of the organic but not the inorganic arsenicals from aqueous solution could be effected by an immiscible solvent. The elaboration of a suitable procedure along these lines for pure solutions containing the four arsenicals, and its application, with some modifications, to urine, is described below.

EXPERIMENTAL

Preliminary investigations

Standard solutions of each arsenic-containing compound were prepared and checked as previously described by Crawford & Levvy (1946). All stages of the fractionations were carried out in separatory funnels and total arsenic in the different fractions was determined after digestion to remove organic matter (Levvy, 1943). Arsenic determinations on the original solutions employed were carried out concurrently with the analyses of samples from the fractionations and the figures so obtained used in calculating the recovery in each fraction; by this procedure constant errors arising from the arsenic determination were to some extent eliminated. Simultaneously with every experiment controls for traces of arsenic in reagents were done and the experimental results corrected accordingly. The arsenic content of every fraction obtained in each separation was ascertained so as to be able to account for all the arsenic originally added. Unless stated to the contrary, experiments were done in triplicate and the results cited are mean values.

Since emulsions were formed with urine, extractions were carried out on dialysates. In some experiments the arsenicals were added to dialysates of normal urine. Rabbit urine was collected in flasks containing 1–2 ml. chloroform for 24 hr. and then dialyzed through 'Visking' synthetic sausage casing against 1–3 vol. distilled water in the refrigerator for 1 day. After the addition of the arsenicals, the dialysate was allowed to stand for 24 hr. in the refrigerator before use. Samples (50 ml.) of the arsenic-containing dialysate were used in the fractionations.

In other experiments, the arsenicals were added directly to urine. Known volumes (10–20 ml.) were dialyzed in the refrigerator for 24 hr. against 50 ml. distilled water containing a few drops of chloroform. Control experiments in which the dialysate and the dialysis sac together with its contents were analyzed for total arsenic at the end of 24 hr. showed this period sufficient for equilibrium across the membrane.

The development of the procedure for separation of the arsenicals in simple solution

Extraction of phenylarsenious acid from aqueous solution by organic solvents. Peroxide-free diethyl ether was found to remove phenylarsenious acid almost quantitatively from aqueous solution acidified to 6N with respect to H_2SO_4, while phenylarsonic acid, arsenious acid and arsenic acid were extracted in negligible amounts under identical conditions (Table 1). The partition coefficient of phenylarsenious acid between ether and 6N-H_2SO_4 was found to be approximately 1·5 and thus several extractions with the solvent were necessary. However, even five extractions with equal volumes of ether always left 5–10% of the arsenical in the aqueous phase. This did not appear to be due to oxidation of the phenylarsenious acid since subsequent extraction with ethane-1:2-dithiol (0·4% v/v) in carbon tetrachloride (the dithiol reagent) removed most of the remaining arsenic.

In these experiments, 40 ml. batches of the arsenical solutions were acidified by the addition of 20 ml. 18N-H_2SO_4 and extracted with five 60 ml. portions of ether, shaking vigorously for 2 min. with each portion of solvent. The ether had been rendered peroxide-free by washing with saturated $FeSO_4$ and then with water. After drying over $CaCl_2$ for at least 12 hr. it was distilled just before use. The combined extracts were evaporated down over distilled water (30 ml.), the last traces of ether being removed *in vacuo*.

Table 1. *Extraction of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid from acidified aqueous solution by peroxide-free ether*

As added (μg.)	As found in ether extracts (μg.)	Added As in first ether extract (%)	As added (μg.)	As found in ether extracts (μg.)
Phenylarsenious acid			Phenylarsonic acid	
77	71·6	56	91	2·7
78	73·4	59	87	1·7
84	75·6	58	93	1·9
83	75·0	63	90	1·8
85	76·5	51	8·8	Nil
86	79·2	61	8·0	Nil
85	78·2	—	Arsenious acid	
85	78·2	—	42	Nil
80	73·5	—	10	1·6
82	77·9	—	7·8	1·2
81	73·0	—	Arsenic acid	
8·8	7·7	—	45	0·9
7·2	5·5	—	11	0·9
			11	0·9
			Mixture of phenylarsonic acid, arsenious acid and arsenic acid	
			110	3·3
			110	Nil

If the ether was not freed from peroxide, partial oxidation of the arsenical occurred as could be shown by its failure to react with the dithiol reagent.

Strong acidification of the aqueous solutions containing phenylarsenious acid in amounts equivalent to 40–80 μg. As appeared to favour the extraction of the arsenical by ether. From 0·2M-phosphate buffer, pH 7, 73% was extracted as against 82 and 93% from 0·01N and 9N-H_2SO_4 respectively.

Other solvents were examined under varying conditions in preliminary experiments. None of these, however, was suitable for separation of phenylarsenious acid from the other arsenicals, phenylarsonic acid, arsenious acid and arsenic acid. Benzene, chloroform, carbon tetrachloride and light petroleum (b.p. 40–60°) removed little or no phenylarsenious acid from aqueous solution. *n*-Butanol and caprylic alcohol, while removing phenylarsenious acid from acid solution, also extracted considerable amounts of the other three arsenic compounds.

Failure to extract phenylarsonic acid quantitatively from aqueous solution by organic solvents. Of the solvents examined, carbon tetrachloride, chloroform and ethyl acetate failed to extract this arsenical from acid solution. 2-Methylbutan-4-ol (primary *iso*-amyl alcohol), while extracting phenylarsonic acid also removed arsenic acid. Caprylic alcohol and *n*-butanol were unsuitable for separation purposes. Contrary to Morgan (1918), ether did not effect any extraction of this compound from acidified aqueous solution (Table 1).

Reduction of the pentavalent arsenicals. As a result of the preliminary work the following procedure for the quantitative separation of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid when present together in aqueous solution was considered. Phenylarsenious acid could be removed by extraction with ether and arsenious acid by extraction with the dithiol reagent (Crawford & Levvy, 1946) leaving in the aqueous phase the two pentavalent compounds. No organic solvent having been found suitable for the separation of the latter, it was planned to reduce these substances and to re-extract the solution with ether to obtain, in the form of phenylarsenious acid, the fraction of the arsenic originally present as phenylarsonic acid. Determination of the arsenic remaining in the aqueous layer, or in subsequent dithiol extracts of the latter would then give the amount of arsenic acid present. This proposed method required the development of a suitable technique for the quantitative reduction of at least phenylarsonic acid, without interference in the later stages of the fractionation, and which, for preference, could be carried out in a separatory funnel.

Klein & Vorhes (1939) recommend the use of a mixture of KI and $Na_2S_2O_3$ in acid solution for the reduction of arsenic acid. On examination, however, this method was found to be quite unsuitable for present purposes since it led to the formation of ether-soluble derivates from both phenylarsonic acid and arsenic acid. This phenomenon was finally shown to be due to the presence of the thiosulphate. When approximately 90 μg. As as phenylarsonic acid or arsenic acid (in 6N-H_2SO_4) were treated with three equivalents of $Na_2S_2O_3$, extraction with ether removed almost all the arsenic in both cases.

SO_2 was next considered as a reducing agent. According to Kurtenacker & Fürstenau (1933), the reduction of arsenic acid by this reagent proceeds at maximal velocity in 0·2–0·26N-H_2SO_4. Experiments showed that SO_2 acting for 45 min. in 0·23N-H_2SO_4 brought about the complete reduction of arsenic acid ($\equiv$100 μg. As), the product being insoluble in ether but easily removed with the dithiol reagent. Under the same conditions, however, phenylarsonic acid was reduced to the extent of only 10%. Increase of the H_2SO_4 concentration before reduction to 6N, the concentration most suitable for the removal of phenylarsenious acid by ether, did not increase the percentage reduction of phenylarsonic acid and greatly decreased that of arsenic acid. The fact that reduction was partial in the case of phenylarsonic acid ruled out the use of SO_2 as a reducing agent.

Attention was again directed to the use in acid solution (6N-H_2SO_4) of KI, alone or in combination with some reagent other than $Na_2S_2O_3$. KI alone gave with arsenic acid a product insoluble in ether but completely extractable by the dithiol reagent provided sufficient of the reducing agent was present (130 mg. for 80 μg. As). From phenylarsonic acid, however, the completely quantitative formation of an ether-soluble derivative was not obtained with that amount of KI which gave complete reduction of arsenic acid. The iodide was added to the arsenical solution (volume 60 ml.) at an initial temperature of 40–60° in a separatory funnel. After cooling for 60 min. the solution was extracted with ether and, in the case of arsenic acid, with the dithiol reagent.

Quantitative formation of an ether-soluble product from phenylarsonic acid and of a product extractable by the dithiol reagent from arsenic acid was obtained when sodium metabisulphite ($Na_2S_2O_5$) was added in addition to the KI. In the case of arsenic acid, the criterion that the reduction product should not be soluble in ether was satisfied (Table 2). In the preliminary experiments, in conformity with Klein & Vorhes's (1939) method of reduction,

Table 2. *Reduction of phenylarsonic acid and arsenic acid in acid solution with a mixture of potassium iodide and sodium metabisulphite*

Initial temp. of solution (°)	As added (μg.)	As removed from aqueous layer (%)	Extraction reagent
		Phenylarsonic acid	
60	84	96	Ether
60	85	99	Ether
18	80	106	Ether
		Arsenic acid	
60	78	Nil	Ether
		100	Dithiol
60	71	Nil	Ether
		95	Dithiol
17	71	101	Dithiol

the temperature of the acidified arsenical solution was approximately 60° at the time of addition of the KI. Further work, however, showed that the reduction of the pentavalent arsenicals went equally well at room temperature. The iodide (130 mg.) was added to the arsenical solution (60 ml.) in a separatory funnel 45 min. before the $Na_2S_2O_5$ (50 mg.), both reagents being in fresh aqueous solution. Extraction with ether, in some cases followed by the dithiol reagent, was done 15 min. later. A pinch of solid metabisulphite was added before extraction with each portion of ether. Reducing the amount of KI used in this process to one-tenth prevented complete reduction of either pentavalent arsenical.

Table 3. *Reduction of phenylarsonic acid and arsenic acid in acid solution with a mixture of potassium iodide and l-ascorbic acid*

As added (μg.)	As removed from aqueous phase (%)	Extraction reagent
	Phenylarsonic acid	
85	95	Ether
87	102	Ether
	Arsenic acid	
78	1	Ether
	99	Dithiol
73	2	Ether
	94	Dithiol

l-Ascorbic acid (50 mg.) also proved suitable in conjunction with KI (130 mg.) for the reduction of phenylarsenic acid and arsenic acid in experiments done at 60° (Table 3). *l*-Ascorbic acid alone failed to bring about reduction of either compound.

The product of the reduction of phenylarsonic acid with KI and $Na_2S_2O_5$ or *l*-ascorbic acid was more readily removed from acid solution by ether than was phenylarsenious acid. The addition of the reducing agents to phenylarsenious acid itself was found to render this substance much more readily extractable by ether. It is possible that the arsenic was extracted as phenyldiiodoarsine (Steinkopf & Smie, 1926).

Other reagents examined for the reduction of both phenylarsonic acid and arsenic acid but without success were formaldehyde, hydrazine sulphate and KBr.

Interference of ethane-1:2-dithiol in the separation of the pentavalent arsenicals after reduction. It was the practice, in the preliminary work, to use one 40 ml. and two 20 ml. portions of 0·4% (v/v) ethane-1:2-dithiol in carbon tetrachloride in the extraction of trivalent arsenic derivatives from acidified aqueous solution (60 ml.). This procedure left sufficient dithiol in the aqueous phase to give an ether-soluble compound with the reduction product of arsenic acid. This difficulty could be overcome either by reducing the quantity of the dithiol reagent and repeatedly extracting the aqueous phase with carbon tetrachloride to remove any residual dithiol, or by adding I_2, as a tincture, to convert the residual dithiol to diethylene tetrasulphide (Fasbender, 1888) and removing the excess I_2 by extraction with carbon tetrachloride. The addition of reducing agents in the next stage of the separation procedure did not appear to regenerate ethane-1:2-dithiol from the diethylene tetrasulphide. In practice it was decided to combine both methods.

Procedure for the quantitative separation of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid in simple solution

The whole fractionation is carried out on one sample of the arsenical solution (40–50 ml.) contained in a separatory funnel of suitable capacity, the stopcock and stopper of which give a tight fit without the use of lubricant.

Twenty ml. 18N-H_2SO_4 are added and the solution shaken vigorously for 2 min. on each occasion with five 60 ml. portions of freshly distilled peroxide-free ether. The combined ether extracts are evaporated over 30 ml. distilled water, the last traces of ether being removed *in vacuo*. The aqueous residue is analyzed for total arsenic, which gives the figure for the phenylarsenious acid content of the original solution.

After the removal of the phenylarsenious acid by ether, the solution is extracted by shaking vigorously for 2 min. on each occasion with two 20 ml. portions of 0·4% (v/v) ethane-1:2-dithiol in carbon tetrachloride, followed by three extractions with 20 ml. portions of carbon tetrachloride. Between each fresh treatment with the dithiol reagent or with carbon tetrachloride, 5 ml. carbon tetrachloride are added to the funnel and run off without any shaking to wash through the stopcock and stem of the funnel. The dithiol and the carbon tetrachloride extracts and washings are combined and the carbon tetrachloride distilled off after the addition of 30 ml. distilled water. The arsenic determination carried out on the aqueous residue so obtained gives the figure corresponding to the arsenious acid content of the original solution.

Before proceeding with the reduction of the pentavalent arsenicals remaining in the aqueous solution, 1 ml. 1% (w/v) iodine in absolute ethanol is added. After 15 min. the solution is shaken with two 20 ml. carbon tetrachloride to remove excess iodine. These extracts may be discarded.

The reduction of the pentavalent arsenicals is then carried out at room temperature by the addition of 1 ml. 13% (w/v) colourless aqueous potassium iodide, followed, after 45 min., by 1 ml. 5% (w/v) freshly prepared sodium metabisulphite solution. Fifteen min. later, the extractions with ether and with the dithiol reagent and carbon tetrachloride are repeated. To ensure the maintenance of complete

Table 4. *Analyses of mixtures of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid in aqueous solution*

Phenylarsenious acid			Arsenious acid			Phenylarsonic acid			Arsenic acid			As found in aqueous residue	Total As		
Added (μg. As)	Found (μg. As)	Error (μg. As)	Added (μg. As)	Found (μg. As)	Error (μg. As)	Added (μg. As)	Found (μg. As)	Error (μg. As)	Added (μg. As)	Found (μg. As)	Error (μg. As)	(μg.)	Added (μg.)	Found (μg.)	Recovery (%)
64·4	67·4	+3·0	6·1	14·1	+8·0	66·4	61·6	−4·8	6·3	5·6	−0·7	Nil	143·2	148·7	104
64·4	65·5	+1·1	6·1	13·9	+7·8	66·4	68·3	+1·9	6·3	5·7	−0·6	Nil	143·2	153·4	107
64·4	61·6	−2·8	6·1	13·2	+7·1	66·4	65·9	−0·5	6·3	6·1	−0·2	2·7	143·2	149·5	105
6·5	5·4	−1·1	61·3	66·2	+4·9	6·1	4·9	−1·2	67·2	61·7	−5·5	7·8	141·1	146·0	104
6·5	6·9	+0·4	61·3	65·8	+4·5	6·1	6·3	+0·2	67·2	64·7	−2·5	3·6	141·1	147·3	105
6·5	10·3	+3·8	61·3	65·4	+4·1	6·1	10·8	+4·7	67·2	62·4	−4·8	4·0	141·1	152·9	109
6·5	6·0	−0·5	12·2	13·2	+1·0	19·9	12·7	−7·2	26·9	21·4	−5·5	Nil	65·5	53·3	81
12·9	13·9	+1·0	18·3	20·8	+2·5	26·6	21·9	−4·7	6·3	Lost	—	Nil	64·1	—	—
19·3	22·1	+2·8	24·4	27·0	+2·6	6·1	4·2	−1·9	13·4	8·2	−5·2	Nil	63·2	61·5	97
25·8	21·1	−4·7	6·1	6·7	+0·6	13·2	11·7	−1·5	20·2	17·3	−2·9	Nil	65·3	56·8	87
25·8	21·2	−4·6	Nil	1·2	+1·2	6·1	6·3	+0·2	Nil	Nil	Nil	Nil	31·9	28·7	90
6·5	4·8	−1·7	Nil	1·0	+1·0	26·6	22·3	−4·3	Nil	Nil	Nil	Nil	33·1	28·1	85

reduction during the ether extractions, a small pinch of solid sodium metabisulphite is added to the solution prior to extraction with each portion of ether. The appearance of a brown colour in the ethereal layer, most noticeable when the extractions are carried out in bright sunlight, may be disregarded. Although the coloration is probably due to traces of iodine formed from hydrogen iodide in the ether by peroxides, it does not appear to interfere in the separation. Arsenic determinations on the ether and on the dithiol extracts give the figures for phenylarsonic acid and arsenic acid respectively in the original solution.

The aqueous phase on the completion of the fractionation should prove on analysis to be arsenic-free if only the four arsenicals specified were present in the original aqueous solution. The method may be curtailed slightly by omitting the final extractions with the dithiol reagent if it is assumed that residual arsenic in the aqueous layer after reduction and extraction with ether corresponds to the arsenic acid originally present.

Results for this procedure using aqueous solutions containing known amounts of the four arsenicals are shown in Table 4, each figure being the result for a single extract. The carbon tetrachloride used to remove excess iodine was also analyzed in each case but no arsenic was ever found in this fraction.

Application of the fractionation procedure to urine dialysate

The formation of ether-soluble compounds between pentavalent arsenicals (phenylarsonic acid and arsenic acid) and urine constituents. The fractionation procedure was unsuitable for urine dialysates owing to the formation of ether-soluble complexes between the pentavalent arsenicals and urinary constituents as a result of which the arsenic corresponding to these compounds appeared in the phenylarsenious acid fraction (Table 5). Attempts to decompose these complexes without change in the valency of the arsenic so that the fractionation could be carried out on one sample of solution were unsuccessful. It was found, however, that an intensification of the reduction process decomposed the complexes. Arsenious acid, or arsenic acid after reduction, was not extracted from urine dialysates by ether and therefore the organic arsenicals could be separated from the inorganic by ether extraction after reduction. Further, complex formation by the pentavalent compounds did not lead to interference in the extraction of the trivalent arsenicals from urine dialysates with the dithiol reagent (Crawford & Levvy, 1946). On the basis of these findings, recourse was made to a method of fractionation involving the use of two samples of the solution.

Table 5. *Formation of ether-soluble complexes from phenylarsonic acid and arsenic acid in the presence of acidified urine dialysate*

Arsenical added to	As added (μg.)	As removed by ether extraction (%)
	Phenylarsonic acid	
Urine	35	89
Urine	42	94
Urine dialysate	66	91
Urine dialysate	93	93
	Arsenic acid	
Urine	34	85
Urine	42	92
Urine dialysate	66	110

Fractionation procedure as applied to urine dialysates containing phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid. Two 40–50 ml. portions of the arsenic-containing urine dialysates are treated in the following manner. The first portion is analyzed after acidification, according to the method described above for pure solution but omitting the initial extractions with ether. The first dithiol extracts thus contain the arsenic corresponding to arsenious acid together with phenylarsenious acid. After reduction by the modified method described below, the arsenic corresponding to phenylarsonic acid and to arsenic acid is removed by extraction with ether and with the dithiol reagent respectively.

The treatment of the second portion of dialysate after acidification commences with reduction. The organic arsenic (phenylarsenious acid and phenylarsonic acid) is removed by ether extraction while the inorganic arsenic (arsenious acid and arsenic acid) is removed by subsequent extraction with the dithiol reagent. The figures for phenylarsonic acid and arsenic acid having been obtained from the fractionation of the first portion of dialysate, those for phenylarsenious acid and arsenious acid may be found by difference.

This modified method has the advantage that possible errors arising from the incomplete extraction of phenylarsenious acid by ether are eliminated since the arsenical is extracted from the dialysate only after the reduction process in which a more readily ether-soluble derivative is formed.

To effect reduction of the pentavalent arsenicals in urine dialysate 2 ml. 13 % (w/v) potassium iodide solution are added and the mixture heated to 55–65°. This heating can be done conveniently by holding the separatory funnel containing the solution in front of an electric radiator. The solution is allowed to cool for 45 min. and 2 ml. 5 % (w/v) sodium metabisulphite solution are added. The extractions with peroxide-free ether are commenced 15 min. later.

The evaporation of the carbon tetrachloride from dithiol extracts should be carried out in the presence

of 30 ml. 1% Na_2CO_3 solution to prevent loss of arsenic due to the volatility of the phenylarsenious acid derivative in steam. The presence of dilute alkali (10–20 drops 10N-NaOH in 30 ml. water) appears to prevent loss of arsenic during the removal of the solvent from the combined ether extracts.

various fractions agreed with the total added with an accuracy within the range of that of the method used for arsenic determination in each fraction (standard deviation 12%; Levvy, 1943).

Modification of the fractionation procedure was necessary for the analysis of urine dialysates owing

Table 6. *Analyses of mixtures of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid added to urine before dialysis, or to urine dialysate*

(Triplicate samples of the dialysate fractionated; averaged results cited.)

Phenylarsenious acid			Arsenious acid			Phenylarsonic acid			Arsenic acid			Total As		
Added (μg. As)	Found (μg. As)	Error (μg. As)	Added (μg. As)	Found (μg. As)	Error (μg. As)	Added (μg. As)	Found (μg. As)	Error (μg. As)	Added (μg. As)	Found (μg. As)	Error (μg. As)	Added (μg. As)	Found (μg. As)	Recovery (%)
Urine dialysate														
27·8	26·0	−1·8	14·4	18·1	+3·7	29·1	27·3	−1·8	15·2	14·7	−0·5	86·5	86·1	100
46·3	48·4	+2·1	23·2	24·2	+1·0	46·7	41·9	−4·8	115·0	113·2	−1·8	231·2	227·7	99
47·4	43·4	−4·0	17·6	20·3	+2·7	46·1	44·8	+3·7	19·0	17·6	−2·4	130·1	131·5	101
Urine														
35·8	33·3	−2·5	17·7	19·2	+1·5	35·2	35·3	+0·1	18·6	17·3	−1·3	107·3	105·1	98
44·8	43·0	−1·8	24·3	24·5	+0·2	43·5	45·8	+2·3	19·1	18·4	−0·7	131·7	131·7	100
38·3	30·6	−7·7	14·3	14·2	−0·1	37·2	39·0	+1·8	15·7	15·1	−0·6	105·5	98·9	94

Table 6 summarizes the results obtained by this procedure in experiments in which mixtures of the four arsenicals were added to urine or urine dialysates. Each experiment was carried in triplicate and the results were averaged before insertion in the table.

DISCUSSION

The fractionation of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid in simple solution was carried out on one sample of the solution in a separatory funnel. Phenylarsenious acid was extracted from the acidified solution by peroxide-free ether, arsenious acid being then removed by extraction with the dithiol reagent (Crawford & Levvy, 1946). Reduction of the arsenic remaining in the aqueous phase by the addition of potassium iodide along with either sodium metabisulphite or *l*-ascorbic acid was followed by a repetition of the ether and dithiol extractions to remove the arsenic corresponding to phenylarsonic acid and arsenic acid respectively. The method was examined over a range of 5–70 μg. arsenic for each compound and the error in any fraction appeared to be constant at an average value of not more than 3 μg. of arsenic. It is of interest to note that, with one exception, only traces ($\gtrless 4$ μg.) of arsenic were found in the aqueous residue from the separation and that the sum of the arsenic contents of the

to the formation in the acidified dialysates of ether-soluble complexes by the pentavalent arsenicals. Complex formation by the pentavalent arsenicals in urine dialysates at normal pH could not be demonstrated by ether extraction but the possibility that the complexes were present as ether-insoluble salts cannot be excluded. The fractionation was carried out with two samples of the dialysate, one of which was submitted to immediate reduction to break down the complexes formed by As^V. The error in the fractionation of urine dialysates was certainly not greater than that in the procedure for simple solutions.

SUMMARY

1. A method is described whereby the amounts of each constituent of a mixture of phenylarsenious acid, phenylarsonic acid, arsenious acid and arsenic acid in simple solution may be determined.

2. Some modification of the method was found to be necessary before it could be applied to urine dialysates since both phenylarsonic acid and arsenic acid were found to yield ether-soluble complexes with urine constituents.

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The Amino-acid Composition of Certain Bacteria and Yeasts

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The amino-acid composition of bacteria has hitherto received little attention; recently Stokes & Gunness (1946) have reported some analyses on a range of bacteria though detailed results are not yet available. Camien, Salle & Dunn (1945) collected values from previous literature and extended our knowledge with regard to a variety of organisms. The analysis of protein hydrolysates by the use of specific decarboxylases (Gale, 1945, 1946) enables accurate values for certain amino-acids to be obtained on comparatively small amounts of material. The present investigation was undertaken in order to determine whether the amino-acid composition of bacterial protein varies with the growth conditions of the organism and also whether there is any marked difference between the amino-acid composition of Gram + and Gram − species.

To determine the amino-acid composition of the bacterial protein it is necessary to correct values obtained with acid-hydrolysates of intact organisms for free amino-acid carried down from the growth medium. Such free amino-acid may be carried down on the surface of the organism or in the internal environment of the organism (Gale, 1947). Taylor (1947) has shown that only Gram + organisms carry down large amounts of free amino-acid in the internal environment, Gram − species containing negligible amounts of free amino-acid.

EXPERIMENTAL

Organisms. The *Esch. coli* used in these experiments was obtained from the National Collection of Type Cultures (N.C.T.C.), No. 86; a Gram − organism N.C.T.C. No. 6578 was isolated by this Unit (Gale & Epps, 1944) also the strains of *B. subtilis*, *Staph. aureus* (B) and *Strep. faecalis*; *Proteus vulgaris*, *Aerobact. aerogenes* and *Staph. aureus* (A) were given to us by the Department of Pathology, Cambridge; *B. brevis* was kindly sent by Dr R. J. Dubos; the three strains of yeast were obtained from the Carlsberg Laboratory through Prof. Winge.

Culture media. Yeasts were cultivated in nutrient salt solution containing 0·2% Difco yeast extract and 4% glucose. For the cultivation of bacteria, a tryptic digest of casein was used as basal medium with the addition of glucose and/or marmite when necessary (Taylor, 1947). *Esch. coli*, *Aerobact. aerogenes*, organism No. 6578 and *Proteus vulgaris* were grown in flasks containing 4·5 l. of medium for each experiment; the other organisms were grown aerobically in liquid media in Roux bottles lying flat. An amino-acid free medium was also used for *Esch. coli* and *Aerobact. aerogenes* and consisted of Stephenson's salt-mixture with the addition of either 1·0% sodium lactate or 1·0% glucose; this was incubated in Roux bottles.

Methods. Amino-acids were estimated by the manometric method using specific amino-acid decarboxylase preparations (Gale, 1945, 1946) and results expressed as amino-acid-N as percentage total-N determined as usual by the micro-Kjedahl method. After growth, the organisms were centrifuged out of the medium and thoroughly washed with water on the centrifuge. The washed organisms were then made up into thick suspension in water (30–50 mg. dry weight/ml.) and the suspension divided into two parts. One part was then hydrolyzed with hydrochloric acid (Gale, 1945) and total amino-acid determined on the hydrolysate. The

other part was placed in a boiling-water bath for 20 min. and amino-acid and N estimations then carried out as usual; values obtained with this portion give the free amino-acid carried down on the surface and in the internal environment of the cells (Gale, 1947). The total amino-acid content of the hydrolysate was then corrected for the free amino-acid content so determined, to give the combined amino-acid liberated by acid hydrolysis.

The free and combined amino-acid content of certain micro-organisms

Table 1 gives typical results obtained for two Gram −, two Gram + organisms and two yeasts grown in casein-digest medium. The free amino-acid buffered media recorded in Table 2 (cf. Gale & Epps, 1942).

Table 2 shows the corrected figures for the amino-acid composition of the bacterial protein of the various organisms used. It can be seen that there was no significant variation with the growth media tested. These results thus confirm the general conclusion of Stokes & Gunness (1946) that the composition of bacterial protein is fixed and characteristic of the organism concerned. There is a suggestion that the lysine content increased with increasing pH at which growth occurred, but it is difficult to say with certainty whether the variation is significant. Table 2 also gives values for *Aerobact. aerogenes* grown under various conditions; again there was no significant variation.

Table 1. *Free and combined amino-acid content of certain micro-organisms*

(Results expressed as % total N.)

Columns: (*a*) total amino-acid estimated on acid hydrolysate of washed suspension; (*b*) free amino-acid estimated on boiled washed suspension; (*c*) combined amino-acid = (*a* − *b*).

Organism ...	*Esch. coli*			*Aerobact. aerogenes*			*Strep. faecalis*		
	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
Arginine-N	10·17	0·10	10·07	10·23	0·32	9·91	6·60	1·14	5·46
Lysine-N	7·80	0·21	7·59	7·42	0·40	7·02	10·50	2·85	7·65
Histidine-N	3·56	0·32	3·24	3·18	0·05	3·13	2·85	0·80	2·05
Tyrosine-N	1·36	0·0	1·36	1·37	0·0	1·37	0·84	0·00	0·84
Glutamic-N	5·92	0·31	5·61	5·48	0·07	5·41	5·93	1·54	4·39

Organism ...	*Staph. aureus*			Dutch top yeast			*Saccharomyces carlsbergensis*		
	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
Arginine-N	5·69	0·72	4·97	12·83	5·04	7·79	10·16	1·59	8·57
Lysine-N	11·06	1·79	9·27	15·05	7·45	7·60	12·0	3·03	8·97
Histidine-N	1·50	0·20	1·30	4·91	1·56	3·35	3·48	0·87	2·61
Tyrosine-N	1·23	0·18	1·05	1·45	0·07	1·38	1·55	0·11	1·44
Glutamic-N	7·53	3·56	3·97	8·12	2·28	5·84	7·27	1·60	5·67

correction is much greater in the case of the Gram + organisms, as these possess a high concentration of certain amino-acids in the internal environment (Taylor, 1947). (Values given in Tables 2 and 3 show corrected figures only.) Since bacterial and yeast cells presumably contain carbohydrate material, there may be a small loss of arginine during hydrolysis procedures (Tristram, 1939).

Effect of growth environment on amino-acid composition of the protein of Esch. coli

The physico-chemical composition of the growth medium may have marked effects on the enzymic composition of an organism such as *Esch. coli* (Gale, 1943) and the present estimations were carried out in order to see whether these changes are reflected in any significant variation in the amino-acid composition of the bacterial protein. *Esch. coli* and *Aerobact. aerogenes* were grown in the various

Amino-acid composition of various bacteria and yeasts

Table 3 gives the corrected values for the amino-acid composition of the protein of a variety of organisms. Generally speaking the analyses show amino-acid contents of the same order for all the organisms tested. When the division of the bacteria into Gram + and Gram − types is considered there is, however, one significant difference, namely in the arginine content. Nine analyses of *Esch. coli* gave a mean arginine content of 10·41 % total N; five analyses of *Aerobact. aerogenes* gave a mean of 10·39 %; single analyses of *P. vulgaris*, organism No. 6578 and *B. brevis* gave 7·75, 12·18 and 9·95 % respectively; an over-all mean value of 10·3 % total N as arginine-N. Comparing these values with five analyses of *Strep. faecalis* giving a mean of 5·4 %; two with *Staph. aureus* giving 4·83 and 4·98 %; and one with *B. subtilis* giving 7·55 % (an over-all average of

Table 2. *Effect of growth environment on combined amino-acid content of the protein of* Esch. coli *and* Aerobact. aerogenes

Values represent the amino-acid content of acid hydrolysates of washed organisms corrected for free amino-acid as shown in Table 1.

Growth media: (1) salt mixture + 1·0 % sodium lactate; (2) salt mixture + 1·0 % glucose; (3) tryptic digest of casein adjusted to pH 8·5; (4), (5), (6), (7), as (3) but adjusted to 8·3, 7·4, 5·5 and 4·9 respectively; (8), (9) tryptic digest of casein + 2·0 % glucose.

(Results expressed as % total N.)

	Esch. coli								
Growth medium ...	1	2	3	4	5	6	7	8	9
Arginine-N	10·50	10·35	10·25	10·35	10·07	10·30	10·25	11·60	10·06
Lysine-N	6·74	—	8·2	7·95	7·6	7·3	6·80	8·0	7·05
Histidine-N	2·97	3·3	3·16	3·32	3·2	3·7	3·03	3·5	3·47
Tyrosine-N	1·23	1·57	1·33	1·57	1·36	1·63	1·23	1·39	1·58
Glutamic-N	5·07	5·57	5·75	5·83	5·60	5·66	5·42	5·90	5·73

	Aerobact. aerogenes				
Growth medium ...	1	3	5	7	8
Arginine-N	10·48	10·70	9·91	10·55	10·3
Lysine-N	6·8	6·48	7·02	6·38	7·2
Histidine-N	3·13	4·0	3·13	3·44	3·32
Tyrosine-N	1·35	1·73	1·37	1·37	1·37
Glutamic-N	5·57	5·80	5·41	5·35	5·85

Table 3. *Combined amino-acid content of various bacteria and yeasts*

The values represent the amino-acid content of acid hydrolysates of washed suspensions of organisms corrected for free amino-acid content as shown in Table 1.

(Results expressed as % total N.)

Gram − bacteria

Organism ...	*Esch. coli*	*Aerobact. aerogenes*	*Proteus vulgaris*	Organism No. 6578	*B. brevis*
No. of estimations ...	9	5	1	1	1
Arginine-N	10·41	10·39	7·75	12·18	9·95
Lysine-N	7·45	6·78	6·39	8·23	7·92
Histidine-N	3·29	3·40	4·39	2·04	2·49
Tyrosine-N	1·43	1·44	2·11	1·68	2·16
Glutamic-N	5·57	5·60	4·88	6·37	7·34

Gram + bacteria

Organism ...	*Strep. faecalis*	*Staph. aureus* (A)	*Staph. aureus* (B)	*B. subtilis*
No. of estimations ...	5	1	1	1
Arginine-N	5·44	4·83	4·98	7·55
Lysine-N	7·61	10·55	9·27	7·52
Histidine-N	2·05	1·15	1·30	2·47
Tyrosine-N	0·84	1·75	1·05	1·49
Glutamic-N	4·39	4·92	3·97	5·29

Yeasts

Organism ...	Yeast foam	Dutch top yeast	*Saccharomyces carlsbergensis*
Arginine-N	8·66	7·79	8·55
Lysine-N	9·07	7·60	6·39
Histidine-N	1·42	3·35	4·39
Tyrosine-N	1·11	1·38	2·11
Glutamic-N	5·50	5·84	4·88

5·57 % total N as arginine-N); it would seem that the Gram + organisms, particularly the Gram + cocci, contained about half the amount of arginine that the Gram − types did. Further confirmation of this can be obtained from the figures given by Camien, Salle & Dunn (1945). These workers used a different method

for the estimation of arginine and when their figures are recalculated as a percentage of total N, they show the arginine content of *Esch. coli* as 15·6, 13·2 and 13·4 % and of *Aerobacter aerogenes* as 13·6 and 13·2 %—a mean value of 13·8 % for the five estimations. These figures can be compared with values obtained for four different *Lactobacilli* (*L. arabinosus*, *L. casei*, *L. pentosus* and *L. fermenti*) of 6·6, 7·2, 6·2 and 9·6 % respectively; the mean value of 7·4 % again represents about half the arginine content of the Gram − species investigated. Our figures would suggest that the *Bacillus* species examined have an arginine content approximately half-way between that of the Gram − coliforms on the one hand and of the Gram + cocci on the other. The yeasts again appear to occupy a similar intermediate position.

SUMMARY

1. Estimation of the combined amino-acid content of micro-organisms involves estimation of the total amino-acid content of acid hydrolysates corrected by a further estimation of the free amino-acid carried down on the surface and in the internal environment of the cells.

2. The amino-acid composition of the protein of *Esch. coli* or *Aerobact. aerogenes* was unaffected by widely varying growth conditions.

3. Values are given for the arginine, lysine, histidine, tyrosine and glutamic acid contents of various bacteria and yeasts. The Gram + cocci examined contained about half as much arginine as the Gram − bacteria.

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ADDENDUM

Since going to press, Stokes, J. L. & Gunness, M. (1946), *J. Bact.* **52**, 195, have published a paper on the amino-acid composition of micro-organisms. They find that the amino-acid composition of an organism is a stable and characteristic property of the cell although striking differences occur between different micro-organisms. They find a significantly higher value for lysine of *Staph. aureus* than *Esch. coli* but no allowance is made for the free lysine in the internal environment of the former. The same difference between the arginine-content of Gram + and Gram − bacteria, as noted in our paper, can be seen in their figures.